	L #	Hits	Search Text	DBs	Time Stamp
1	L1	87	dna adj (pk or activated adj (protein adj kinase$1 or pk))	USPAT; US-PGPUB	2003/06/04 14:11
2	L2	25	1 same (substrate$ or peptide$)	USPAT; US-PGPUB	2003/06/04 14:18
3	L3	19	1 same (assay$8 or detect$8 or quantit$8)	USPAT; US-PGPUB	2003/06/04 14:20
4	L4	6232	p53	USPAT; US-PGPUB	2003/06/04 14:23
5	L5	505	4 near6 (fragment$4 or peptide$ or portion$)	USPAT; US-PGPUB	2003/06/04 14:27
6	L6	971	4 near4 human	USPAT; US-PGPUB	2003/06/04 14:27
7	L7	279	5 and 6	USPAT; US-PGPUB	2003/06/04 15:11
8	L8	471	4 same (phosphorylat$ or kinase$1 or termin$8) same (fragment$4 or peptide$ or portion$)	USPAT; US-PGPUB	2003/06/04 15:14
9	L9	130	8 and 7	USPAT; US-PGPUB	2003/06/04 15:14
10	L10	12	5 and 1	USPAT; US-PGPUB	2003/06/04 16:21

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	87	dna adj (pk or activated adj (protein adj kinase$1 or pk))	USPAT; US-PGPUB	2003/06/04 14:11
2	L2	25	1 same (substrate$ or peptide$)	USPAT; US-PGPUB	2003/06/04 14:12

PGPUB-DOCUMENT-NUMBER: 20030054421

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030054421 A1

TITLE: Nucleic acids, proteins, and antibodies

PUBLICATION-DATE: March 20, 2003

US-CL-CURRENT: 435/7.23, 435/183 , 435/320.1 , 435/325 , 435/6 , 435/69.1
        , 536/23.2

APPL-NO: 10/ 102806

DATE FILED: March 22, 2002

RELATED-US-APPL-DATA:

 child 10102806 A1 20020322

 parent continuation-of 09925298 20010810 US PENDING

 child 09925298 20010810 US

 parent continuation-in-part-of PCT/US00/05881 20000308 US UNKNOWN

 non-provisional-of-provisional 60124270 19990312 US

PGPUB-DOCUMENT-NUMBER: 20030022263

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030022263 A1

TITLE: ATM kinase modulation for screening and therapies

PUBLICATION-DATE: January 30, 2003

US-CL-CURRENT: 435/15

APPL-NO: 10/ 024123

DATE FILED: December 17, 2001

RELATED-US-APPL-DATA:

  child 10024123 A1 20011217

  parent division-of 09400653 19990921 US PATENTED

  child 09400653 19990921 US

  parent continuation-in-part-of 09248061 19990210 US PENDING

[0001] This is a division of application Ser. No. 09/400,653, filed Sep. 21, 1999, which is a continuation-in-part of and claims the priority of U.S. application Ser. No. 09/248,061 filed Feb. 10, 1999. Each of these prior applications is hereby incorporated herein by reference, in its entirety.

[0001] This application claims the benefit of U.S. Provisional Application No. 60/218,498, filed Jul. 14, 2000, the content of which is hereby incorporated by reference.

PGPUB-DOCUMENT-NUMBER: 20020165218

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020165218 A1

TITLE: Materials and methods to potentiate cancer treatment

PUBLICATION-DATE: November 7, 2002

US-CL-CURRENT: 514/210.2, 514/217.05 , 514/217.06 , 514/242 , 514/252.01
, 514/252.03 , 514/252.05 , 514/256 , 514/84 , 514/85
, 544/182 , 544/232 , 544/238 , 544/243 , 544/319 , 544/328

APPL-NO: 09/ 941897

DATE FILED: August 28, 2001

RELATED-US-APPL-DATA:

 non-provisional-of-provisional 60229899 20000901 US

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of provisional U.S. Patent
Application No. 60/229,899, filed Sep. 1, 2000.

PGPUB-DOCUMENT-NUMBER: 20020151030

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020151030 A1

TITLE: Claspin proteins and methods of use thereof

PUBLICATION-DATE: October 17, 2002

US-CL-CURRENT: 435/226, 435/320.1 , 435/325 , 435/69.1 , 536/23.2

APPL-NO: 09/ 982091

DATE FILED: October 17, 2001

RELATED-US-APPL-DATA:

 non-provisional-of-provisional 60241246 20001017 US

RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C.
.sctn.119(e) of U.S. Provisional Application No. 60/241,246, filed Oct. 17,
2000, incorporated herein in its entirety.

PGPUB-DOCUMENT-NUMBER: 20020150885

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020150885 A1

TITLE: Novel fluorogenic or fluorescent reporter molecules and their applications for whole-cell fluorescence screening assays for caspases and other enzymes and the use thereof

PUBLICATION-DATE: October 17, 2002

US-CL-CURRENT: 435/5

APPL-NO: 09/ 947387

DATE FILED: September 7, 2001

RELATED-US-APPL-DATA:

child 09947387 A1 20010907

parent division-of 09168888 19981009 US PATENTED

non-provisional-of-provisional 60145746 19980303 US

non-provisional-of-provisional 60061582 19971010 US

[0001] This application is a claims benefit of priority under 35 U.S.C. .sctn. 365(c) and .sctn. 120 to International Application Number PCT/US00/05881, filed Mar. 8, 2000 which was published by the International Bureau in the English language as International Publication Number WO00/55173 on Sep. 21, 2000 and under 35 U.S.C. .sctn. 119(e) to U.S. application No. 60/124,270 filed March 12, 1999, both of which are hereby incorporated by reference herein.

PGPUB-DOCUMENT-NUMBER: 20020022231

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020022231 A1

TITLE: Method for identifying modulators of DNA structure-specific binding proteins by high-throughput screening

PUBLICATION-DATE: February 21, 2002

US-CL-CURRENT: 435/6

APPL-NO: 09/ 931883

DATE FILED: August 20, 2001

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60226441 20000818 US

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of the provisional patent application U.S. Ser. No. 60/226,441 filed Aug. 18, 2000, which is incorporated by reference in its entirety.

US-PAT-NO: 6573364

DOCUMENT-IDENTIFIER: US 6573364 B1

TITLE: Isolation and characterization of Hermansky Pudlak Syndrome (HPS) protein complexes and HPS protein-interacting proteins

DATE-ISSUED: June 3, 2003

US-CL-CURRENT: 530/350, 435/317.1

APPL-NO: 09/ 266225

DATE FILED: March 10, 1999

US-PAT-NO: 6534056

DOCUMENT-IDENTIFIER: US 6534056 B1

TITLE: Therapeutic and diagnostic uses of protein tyrosine
                phosphatase TC-PTP

DATE-ISSUED: March 18, 2003

US-CL-CURRENT: 424/146.1, 435/4 , 435/7.1

APPL-NO: 09/ 466992

DATE FILED: December 10, 1999

PARENT-CASE:

   CROSS-REFERENCE TO RELATED APPLICATIONS

   The application claims priority to U.S. provisional patent application No.
60/111,993, filed Dec. 11, 1998.

US-PAT-NO: 6521456

DOCUMENT-IDENTIFIER: US 6521456 B1

TITLE: Cellular transport system for the transfer of a nucleic
                acid through the nuclear envelope and methods thereof

DATE-ISSUED: February 18, 2003

US-CL-CURRENT: 435/455, 435/320.1 , 436/71 , 530/350

APPL-NO: 09/ 869875

DATE FILED: July 6, 2001

PARENT-CASE:

   This application claims benefit of International Application No.
PCT/DE00/00061, filed Jan. 3, 2000; which claims priority of German
Applications No. 199 33 939.2, filed on Jul. 20, 1999 and 199 00 513.3 filed
on Jan. 8, 1999. The contents of all of the foregoing applications in their
entireties are incorporated by reference into the present application.

                FOREIGN-APPL-PRIORITY-DATA:
   COUNTRY   APPL-NO   APPL-DATE
   DE   199 00 513   January 8, 1999
   DE   199 33 939   July 20, 1999

PCT-DATA:
 APPL-NO: PCT/DE00/00061
 DATE-FILED: January 3, 2000
 PUB-NO: WO00/40742
 PUB-DATE: Jul 13, 2000
 371-DATE:
 102(E)-DATE:

US-PAT-NO: 6441158

DOCUMENT-IDENTIFIER: US 6441158 B1

TITLE: Oligomers that bind to ku protein

DATE-ISSUED: August 27, 2002

US-CL-CURRENT: 536/24.5, 536/23.1

APPL-NO: 09/ 223139

DATE FILED: December 30, 1998

PARENT-CASE:

  CROSS-REFERENCE TO RELATED APPLICATIONS

  This application claims benefit of U.S. provisional application No. 60/070,278, filed Dec. 31, 1997.

US-PAT-NO: 6348311

DOCUMENT-IDENTIFIER: US 6348311 B1
See image for Certificate of Correction

TITLE: ATM kinase modulation for screening and therapies

DATE-ISSUED: February 19, 2002

US-CL-CURRENT: 435/5, 435/15

APPL-NO: 09/ 400653

DATE FILED: September 21, 1999

PARENT-CASE:

   This application is a continuation in part of U.S. application No. 09/248,
061 filed 02/10/1999 pending.

US-PAT-NO: 6342611

DOCUMENT-IDENTIFIER: US 6342611 B1

TITLE: Fluorogenic or fluorescent reporter molecules and their
                  applications for whole-cell fluorescence screening assays
                  for capsases and other enzymes and the use thereof

DATE-ISSUED: January 29, 2002

US-CL-CURRENT: 549/227

APPL-NO: 09/ 168888

DATE FILED: October 9, 1998

PARENT-CASE:

  This application claims benefit of provisional application Ser. No.
60/061,582 filed Oct. 10, 1997.

US-PAT-NO: 6335429

DOCUMENT-IDENTIFIER: US 6335429 B1

TITLE: Fluorogenic or fluorescent reporter molecules and their
              applications for whole-cell fluorescence screening assays
              for caspases and other enzymes and the use thereof

DATE-ISSUED: January 1, 2002

US-CL-CURRENT: 530/402, 435/18 , 435/24 , 436/546

APPL-NO: 09/ 521650

DATE FILED: March 8, 2000

PARENT-CASE:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a division of Appl. Ser. No. 09/168,888, filed Oct.
9, 1998, which claims the benefit of U.S Prov. Appl. No. 60/145,746, filed
Mar. 3, 1998, abandoned, and U.S. Prov. Appl. No. 60/061,582, filed Oct.
10, 1997, abandoned.

US-PAT-NO: 6331396

DOCUMENT-IDENTIFIER: US 6331396 B1

TITLE: Arrays for identifying agents which mimic or inhibit the
                  activity of interferons

DATE-ISSUED: December 18, 2001

US-CL-CURRENT: 435/6, 435/287.2 , 536/23.1 , 536/23.52 , 536/24.3
         , 536/24.31

APPL-NO: 09/ 405438

DATE FILED: September 23, 1999

PARENT-CASE:

  This application claims the benefit of U.S. Provisional No. 60/101,497,
filed Sep. 23, 1998.

US-PAT-NO: 6248904

DOCUMENT-IDENTIFIER: US 6248904 B1

TITLE: Fluorescence dyes and their applications for whole-cell
                  fluorescence screening assays for caspases, peptidases,
                  proteases and other enzymes and the use thereof

DATE-ISSUED: June 19, 2001

US-CL-CURRENT: 549/227, 435/15 , 435/23 , 435/24 , 436/93

APPL-NO: 09/ 357952

DATE FILED: July 21, 1999

PARENT-CASE:

   The present application claims the benefit of U.S. provisional application
60/093,642 filed Jul. 21, 1998 abandoned.

US-PAT-NO: 6171857

DOCUMENT-IDENTIFIER: US 6171857 B1
See image for Certificate of Correction

TITLE: Leucine zipper protein, KARP-1 and methods of regulating
              DNA dependent protein kinase activity

DATE-ISSUED: January 9, 2001

US-CL-CURRENT: 435/325, 435/252.1 , 435/320.1 , 536/23.1 , 536/23.5
              , 536/24.3 , 536/24.31 , 536/24.33

APPL-NO: 09/ 173914

DATE FILED: October 16, 1998

PARENT-CASE:

   RELATED APPLICATIONS

   This application claims priority under 35 USC .sctn. 119(e) from U.S.
Provisional Patent Application Ser. No. 60/064,557 filed on Oct. 17, 1997,
entitled A NOVEL LEUCINE ZIPPER PROTEIN, KARP-1 AND METHODS OF REGULATING
DNA
DEPENDENT PROTEIN KINASE ACTIVITY. The content of the provisional application
is hereby expressly incorporated by reference.

US-PAT-NO: 6025194

DOCUMENT-IDENTIFIER: US 6025194 A

TITLE: Nucleic acid sequence of senescence asssociated gene

DATE-ISSUED: February 15, 2000

US-CL-CURRENT: 435/320.1, 435/325 , 536/23.1 , 536/23.5 , 536/24.1

APPL-NO: 08/ 974180

DATE FILED: November 19, 1997

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	87	dna adj (pk or activated adj (protein adj kinase$1 or pk))	USPAT; US-PGPUB	2003/06/04 14:11
2	L2	25	1 same (substrate$ or peptide$)	USPAT; US-PGPUB	2003/06/04 14:18
3	L3	19	1 same (assay$8 or detect$8 or quantit$8)	USPAT; US-PGPUB	2003/06/04 14:20

PGPUB-DOCUMENT-NUMBER: 20030096262

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030096262 A1

TITLE: Uses of Ku70

PUBLICATION-DATE: May 22, 2003

US-CL-CURRENT: 435/6

APPL-NO: 10/ 161025

DATE FILED: June 3, 2002

RELATED-US-APPL-DATA:

  child 10161025 A1 20020603

  parent division-of 09343634 19990630 US GRANTED

  parent-patent 6399298 US

  non-provisional-of-provisional 60091188 19980630 US

[0001] This application claims the benefit of U.S. provisional Application No. 60/091,188, filed Jun. 30, 1998. The contents of the preceding application are hereby incorporated into this application by reference.

[0001] This application claims the benefit of U.S. Provisional application No. 60/091,181, filed Jun. 30, 1998, the content of which is hereby incorporated by reference.

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. provisional application Serial No. 60/273,124, filed Mar. 2, 2001.

PGPUB-DOCUMENT-NUMBER: 20030022263

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030022263 A1

TITLE: ATM kinase modulation for screening and therapies

PUBLICATION-DATE: January 30, 2003

US-CL-CURRENT: 435/15

APPL-NO: 10/ 024123

DATE FILED: December 17, 2001

RELATED-US-APPL-DATA:

  child 10024123 A1 20011217

  parent division-of 09400653 19990921 US PATENTED

  child 09400653 19990921 US

  parent continuation-in-part-of 09248061 19990210 US PENDING

[0001] This is a division of application Ser. No. 09/400,653, filed Sep. 21, 1999, which is a continuation-in-part of and claims the priority of U.S. application Ser. No. 09/248,061 filed Feb. 10, 1999. Each of these prior applications is hereby incorporated herein by reference, in its entirety.

[0001] This application claims the benefit of U.S. Provisional Application No. 60/218,498, filed Jul. 14, 2000, the content of which is hereby incorporated by reference.

PGPUB-DOCUMENT-NUMBER: 20020165218

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020165218 A1

TITLE: Materials and methods to potentiate cancer treatment

PUBLICATION-DATE: November 7, 2002

US-CL-CURRENT: 514/210.2, 514/217.05 , 514/217.06 , 514/242 , 514/252.01
, 514/252.03 , 514/252.05 , 514/256 , 514/84 , 514/85
, 544/182 , 544/232 , 544/238 , 544/243 , 544/319 , 544/328

APPL-NO: 09/ 941897

DATE FILED: August 28, 2001

RELATED-US-APPL-DATA:

 non-provisional-of-provisional 60229899 20000901 US

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of provisional U.S. Patent
Application No. 60/229,899, filed Sep. 1, 2000.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of the provisional patent application U.S. Ser. No. 60/226,441 filed Aug. 18, 2000, which is incorporated by reference in its entirety.

PGPUB-DOCUMENT-NUMBER: 20010036929

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20010036929 A1

TITLE: Xrcc3 is required for assembly of Rad51-complexes in vivo

PUBLICATION-DATE: November 1, 2001

US-CL-CURRENT: 514/44, 424/649 , 514/34

APPL-NO: 09/ 844538

DATE FILED: April 26, 2001

RELATED-US-APPL-DATA:

child 09844538 A1 20010426

parent division-of 09404053 19990922 US PENDING

non-provisional-of-provisional 60101909 19980925 US

US-PAT-NO: 6455250

DOCUMENT-IDENTIFIER: US 6455250 B1

TITLE: Endonuclease compositions and methods of use

DATE-ISSUED: September 24, 2002

US-CL-CURRENT: 435/6, 424/93.2 , 424/93.21 , 435/320.1 , 435/325 , 435/455
        , 435/456 , 435/69.1 , 536/23.2 , 536/23.5

APPL-NO: 09/ 210422

DATE FILED: December 11, 1998

PARENT-CASE:

  This application claims priority under 35 U.S.C. 119 (e) of provisional
U.S. patent application Ser. No. 60/069,205, filed Dec. 11, 1997.

US-PAT-NO: 6441158

DOCUMENT-IDENTIFIER: US 6441158 B1

TITLE: Oligomers that bind to ku protein

DATE-ISSUED: August 27, 2002

US-CL-CURRENT: 536/24.5, 536/23.1

APPL-NO: 09/ 223139

DATE FILED: December 30, 1998

PARENT-CASE:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. provisional application No. 60/070,278, filed Dec. 31, 1997.

US-PAT-NO: 6399298

DOCUMENT-IDENTIFIER: US 6399298 B1

TITLE: Ku70--related methods

DATE-ISSUED: June 4, 2002

US-CL-CURRENT: 435/6, 435/7.1 , 435/91.2

APPL-NO: 09/ 343634

DATE FILED: June 30, 1999

PARENT-CASE:

This application claims the benefit of U.S. provisional Application No. 60/091,188, filed Jun. 30, 1998. The contents of the preceding application are hereby incorporated into this application by reference.

US-PAT-NO: 6387640

DOCUMENT-IDENTIFIER: US 6387640 B1

TITLE: ATM kinase modulation for screening and therapies

DATE-ISSUED: May 14, 2002

US-CL-CURRENT: 435/15, 435/194 , 435/252.3 , 435/320.1 , 435/325

APPL-NO: 09/ 248061

DATE FILED: February 10, 1999

US-PAT-NO: 6348311

DOCUMENT-IDENTIFIER: US 6348311 B1
See image for Certificate of Correction

TITLE: ATM kinase modulation for screening and therapies

DATE-ISSUED: February 19, 2002

US-CL-CURRENT: 435/5, 435/15

APPL-NO: 09/ 400653

DATE FILED: September 21, 1999

PARENT-CASE:

   This application is a continuation in part of U.S. application No. 09/248, 061 filed 02/10/1999 pending.

US-PAT-NO: 6242175

DOCUMENT-IDENTIFIER: US 6242175 B1

TITLE: Methods and means relating to retrotransposon and
              retroviral integration

DATE-ISSUED: June 5, 2001

US-CL-CURRENT: 435/5, 424/94.1 , 435/183 , 435/412 , 435/6 , 435/70.1
    , 536/23.1

APPL-NO: 09/ 341515

DATE FILED: July 12, 1999

PARENT-CASE:

CROSS-REFERENCE TO RELATED APPLICATIONS

   This application is a .sctn.371 National Stage filing of International
Patent application no. PCT/GB98/00099, filed Jan. 13, 1998, which claims
priority to Great Britain patent application no. GB9700574.8, filed Jan. 13,
1997.

              FOREIGN-APPL-PRIORITY-DATA:
   COUNTRY   APPL-NO   APPL-DATE
   GB   9700574   January 13, 1997

PCT-DATA:
 APPL-NO: PCT/GB98/00099
 DATE-FILED: January 13, 1998
 PUB-NO: WO98/30903
 PUB-DATE: Jul 16, 1998
 371-DATE: Jul 12, 1999
 102(E)-DATE:Jul 12, 1999

US-PAT-NO: 6200754

DOCUMENT-IDENTIFIER: US 6200754 B1

TITLE: Inhibitors of alternative alleles of genes encoding products that mediate cell response to environmental changes

DATE-ISSUED: March 13, 2001

US-CL-CURRENT: 435/6, 435/375 , 536/23.1 , 536/24.5

APPL-NO: 09/ 045054

DATE FILED: March 19, 1998

US-PAT-NO: 6171857

DOCUMENT-IDENTIFIER: US 6171857 B1
See image for Certificate of Correction

TITLE: Leucine zipper protein, KARP-1 and methods of regulating
                DNA dependent protein kinase activity

DATE-ISSUED: January 9, 2001

US-CL-CURRENT: 435/325, 435/252.1 , 435/320.1 , 536/23.1 , 536/23.5
                , 536/24.3 , 536/24.31 , 536/24.33

APPL-NO: 09/ 173914

DATE FILED: October 16, 1998

PARENT-CASE:

   RELATED APPLICATIONS

   This application claims priority under 35 USC .sctn. 119(e) from U.S.
Provisional Patent Application Ser. No. 60/064,557 filed on Oct. 17, 1997,
entitled A NOVEL LEUCINE ZIPPER PROTEIN, KARP-1 AND METHODS OF REGULATING
DNA
DEPENDENT PROTEIN KINASE ACTIVITY. The content of the provisional application
is hereby expressly incorporated by reference.

US-PAT-NO: 6132968

DOCUMENT-IDENTIFIER: US 6132968 A

TITLE: Methods for quantitating low level modifications of
                 nucleotide sequences

DATE-ISSUED: October 17, 2000

US-CL-CURRENT: 435/6, 435/7.1

APPL-NO: 09/ 078347

DATE FILED: May 13, 1998

US-PAT-NO: 5955644

DOCUMENT-IDENTIFIER: US 5955644 A

TITLE: Ku deficient cells and non-human transgenic animals

DATE-ISSUED: September 21, 1999

US-CL-CURRENT: 800/18, 435/325 , 435/354 , 435/440 , 435/455

APPL-NO: 08/ 695866

DATE FILED: August 8, 1996

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	87	dna adj (pk or activated adj (protein adj kinase$1 or pk))	USPAT; US-PGPUB	2003/06/04 14:11
2	L2	25	1 same (substrate$ or peptide$)	USPAT; US-PGPUB	2003/06/04 14:18
3	L3	19	1 same (assay$8 or detect$8 or quantit$8)	USPAT; US-PGPUB	2003/06/04 14:20
4	L4	6232	p53	USPAT; US-PGPUB	2003/06/04 14:23
5	L5	505	4 near6 (fragment$4 or peptide$ or portion$)	USPAT; US-PGPUB	2003/06/04 14:27
6	L6	971	4 near4 human	USPAT; US-PGPUB	2003/06/04 14:27
7	L7	279	5 and 6	USPAT; US-PGPUB	2003/06/04 15:11
8	L8	471	4 same (phosphorylat$ or kinase$1 or termin$8) same (fragment$4 or peptide$ or portion$)	USPAT; US-PGPUB	2003/06/04 15:14
9	L9	130	8 and 7	USPAT; US-PGPUB	2003/06/04 15:14

PGPUB-DOCUMENT-NUMBER: 20030086935

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030086935 A1

TITLE: p53 vaccine

PUBLICATION-DATE: May 8, 2003

INVENTOR-INFORMATION:
NAME   CITY   STATE   COUNTRY   RULE-47
Levine, Arnold J.   Princeton   NJ   US

APPL-NO: 09/ 946549

DATE FILED: September 6, 2001

RELATED-US-APPL-DATA:

  child 09946549 A1 20010906

  parent division-of 08366512 19941230 US ABANDONED

  child 08366512 19941230 US

  parent continuation-in-part-of 08185738 19940124 US ABANDONED

  child 08185738 19940124 US

  parent continuation-in-part-of 08015493 19930209 US ABANDONED

  child 08015493 19930209 US

  parent continuation-in-part-of 07918292 19920722 US ABANDONED

US-CL-CURRENT: 424/185.1, 424/277.1

ABSTRACT:

The subject invention provides a vaccine composition comprising a mutant or wild-type p53 protein in a form that, when presented to the immune system of a mammal, induces an effective immune response, i.e., either on the surface of an antigen presenting cell or combined with a pharmaceutically acceptable adjuvant. Further, the subject invention provides a method of inhibiting the growth of tumors in mammals comprising treating a mammal with an · immunologically effective amount of a vaccine comprising the mutant or wild-type p53 protein.

[0001] This application is a continuation-in-part of Ser. No. 08/185,738, filed Jan. 24, 1994, which is a continuation-in-part of Ser. No. 08/015,493,

filed Feb. 9, 1993, which in turn is a continuation-in-part of Ser. No. 07/918,292, filed Jul. 22, 1992, both of which are incorporated herein by reference.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX (5):

[0006] Approximately 10-20% of humans with cancers have tumors that produce antibodies directed against the p53 protein; de Fromentel et al., International Journal of Cancer 39, 185-189 (1987); Crawford et al., International Journal of Cancer 30, 403-408 (1982). The presence of these antibodies suggests that class 11 receptors of the human HLA or the murine H-2 locus can present **peptide antigens of p53** to the CD-4 helper T-cell and B-cell system, resulting in an immune response. Antibodies are not, however, believed to be effective ant-tumor agents. Therefore, the presence of ant-p53 antibodies in humans with cancer does not suggest the possibility of cancer patents producing an effective anti-tumor immune response.

Brief Description of Drawings Paragraph - DRTX (7):

[0017] FIG. 6: Immunoprecipitation of in vitro translated **human p53** using 3 .mu.l of pooled serum drawn from mice shown in FIG. 4 at the time of injection. The autoradiograph of the SDS-PAGE of the immunoprecipitates is shown. Lane 1: immunoprecipitation of p53 and p53 breakdown products using monoclonal antibody pab421; lane 2,3,4,5: immunoprecipitation with serum from nonimmunized mice, (10)3-273.1NT24, (10)3, (10)3-273.1 immunized mice respectively; lane 6 [.sup.14C]-labeled molecular weight markers (BRL).

Brief Description of Drawings Paragraph - DRTX (8):

[0018] FIG. 7: FACS-analysis of MHC-1 H2-K.sup.d or H2-K.sup.b surface expression on (10)3, (10)3-273.1, (10)3-273.1N124 cells. P815 and P815:273 cells are used as controls. They are mouse mastocytoma cell lines and express high levels of H2-K.sup.d. P815:273 express the **human p53** allele mutated at amino acid 273.

Brief Description of Drawings Paragraph - DRTX (11):

[0021] FIG. 10: Immunoblot analysis of p53 protein expressed in BCG bacteria. Bacteria were lysed in cytoplasmic lysis buffer. One mg of protein extract was analyzed in each experimental lane of this SDS-PAGE. The protein was transferred to nitrocellulose, incubated with antibody pab421 and peroxidase conjugated goat anti-mouse antibody (1:5000, Cappel), and developed with ECL (Amersham). One-hundred ng **human p53** protein purified from baculovirus extract was used as a positive control. Two out of two clones of wild-type and mutant p53 exon 5-11 express the respective 28 kD fragment. The upper bands might represent aggregates and are present at [fraction (1/10)] the level of the specific expression product. One clone (lane 2) of the full-length p53 expression constructs expresses p53.

Brief Description of Drawings Paragraph - DRTX (12):

[0022] FIG. 11: Immunoprecipitation and subsequent Western analysis of recombinant **human full-length p53** expressed in BCG bacteria. Bacteria were lysed in lysis buffer and 1 mg extract was immunoprecipitated using either pab421, pab1801 or a control antibody pab419. The immunoprecipitable material was subjected to SDS-PAGE analysis, transferred to nitrocellulose and detected with polyclonal rabbit anti-p53 antiserum (1:500 diluton) and visualized with peroxidase conjugated anti-rabbit-IgG (Cappel, 1:5000 diluton) and ECL (Amersham). BCG-SN.sub.3 expressed p53 under control of the BCG hsp60 promoter. p53 was immunoprecipitable by pabl801 directed against an N-terminal epitope and pab421 directed against a C-terminal epitope. Heat-shock of the bacteria did not increase the expression level. Untransformed BCG bacteria did not express p53. The secondary anti-rabbit-IgG had cross-reactivity to the IgG heavy and light chains of the monoclonal antibodies. More p53 was immunoprecipitated with pab421, which is the p53 antibody with the highest binding constant to p53 and which recognizes native and also denatured protein.

Brief Description of Drawings Paragraph - DRTX (15):

[0025] FIG. 14: Immunization with ALVAC virus. Shown on the vertical axis is the time in days until the mice immunized with the respective vaccine (on the horizontal axis) developed tumors. Open circles represent individual mice, open squares the mean tumor-free survival time within each group.+-.standard deviation. The lower p-values show the significance as calculated using a Mann-Whitney U Test between each subgroup and the vector alone. The upper p-values show the significance in tumor-free survival time compared to immunization with ALVAC vector alone for the groups of mice immunized with ALVAC expressing either **human or murine p53** as calculated using a Mann-Whitney U Test.

Detail Description Paragraph - DETX (4):

[0028] The p53 protein may be mutated. The data shown in Example 1 demonstrate that overexpression of mutant p53 in experimental tumors can induce an immune response which is dependent on mutant p53 protein expression. This immune response constrained tumor growth of moderately tumorigenic cells ((10)3-273.1) and upon immunization resulted in tumor rejection of highly tumorigenic variants (10)3-273.1 NT24). Tolerance to **p53 evoked by expression of a human p53** transgene impaired tumor immunity.

Detail Description Paragraph - DETX (6):

[0030] Suitable mutant **human p53** genes are described in Levine, A. J. et al., The p53 Tumor Suppressor Gene, Nature 351:453456 (1991). Most of the point mutations that occur in the p53 gene are missense mutations, giving rise to an altered p53 protein. The majority of mutations are clustered between amino-acid residues 130 and 290, and mostly localized in four "hot spot" regions of the protein, which coincide with the four most highly conserved regions of the p53 gene; see Nigro et al, Nature 342, 705-708 (1989). The four "hot spot" mutation regions are at codons 132-143; 174-179; 236-248; and 272-281. The frequency and distribution of these hot spots differ among cancers from different tissue types.

Detail Description Paragraph - DETX (8):

[0032] The entire **p53 gene or fragments of the p53** gene may, for example, be isolated by using the known DNA sequence to construct oligonucleotide probes. To do so, DNA restriction fragments are identified by Southern hybridization using labelled oligonucleotide probes derived from the known sequence.

Detail Description Paragraph - DETX (13):

[0037] The mutant or wild-type **p53 protein fragment** preferably contains sufficient amino acid residues to define an epitope of the antigen. The fragment may, for example, be a minigene encoding only the epitope. Methods for isolating and identifying immunogenic fragments from known immunogenic proteins are described by Salfeld et al. in J. Virol. 63, 798-808 (1989) and by Isola et al. in J. Virol. 63, 2325-2334 (1989).

Detail Description Paragraph - DETX (14):

[0038] The wild-type or mutant **p53 protein fragments** may be expressed by truncated wild- or type or mutant p53 genes. The p53 gene is composed of 11 exons. The first exon (213 bp) is non-coding and is located 8-10 Kb away from the second exon which contains the translational start codon. In the present invention, the truncated p53 genes encoding wild- or type or mutant **p53 protein fragments** may lack any one exon or more than one exon, or any portion thereof. The number of exons lacking from the truncated p53 genes encoding wild-type or mutant **p53 protein fragments** preferably lack 2-4 exons, and more preferably, lack any of the first 4 exons. In another preferred embodiment, the truncated p53 genes lack all first 4 exons, and thereby comprise exons 5-11.

Detail Description Paragraph - DETX (32):

[0056] The mutant or wild-type p53 protein may also be presented to the immune system on the surface of recombinant bacterial cells. A suitable recombinant bacterial cell is an avirulent strain of Mycobacterium bovis, such as bacille Calmette-Guerin (BCG), or an avirulent strain of Salmonella, such as S. typhimurium. The recombinant bacterial cells may be prepared by cloning DNA comprising the active **portion of the p53** protein in an avirulent strain, as is known in the art; see, for example, Curtiss et al., Vaccine 6, 155-160 (1988) and Galan et al., Gene 94, 29-35 (1990) for preparing recombinant Salmonella and Stover, C. K. et al., Vaccines 91, Cold Spring Harbor Laboratory Press, pp. 393-398 (1991) for preparing recombinant BCG.

Detail Description Paragraph - DETX (36):

[0060] As shown in Examples 2-3, immunization with recombinant BCG or recombinant ALVAC vaccines expressing **p53 peptide sequences protected mice against challenge with a p53** expressing tumor cell line.

Detail Description Paragraph - DETX (52):

[0071] For the analysis of serum antibody levels, mice were bled and the blood was left at 40.degree. C. ON. The blood-clot was pelleted and the serum

stored at -80.degree. C. For analysis, 5 .mu.l serum were diluted into 50 .mu.l PBS and 10 .mu.l of this mix were incubated ON at 4.degree. C. with 500 .mu.l [35S]-methionine labeled cell-extract from SV80 cells, which are SV40 transformed human fibroblasts and which express high levels of p53 and SV40 T antigen, in cytoplasmic lysis buffer (CLB) (10 mM Tris pH 7.4, 250 mM sucrose, 160 mM KCl, 50 mM E-amino-caproic acid, 0.5% NP-40 supplemented to 3 mM 13-mercaptoethanol, 1 mM PMSF and 0.28 TIU/ml aprotinin immediately prior to use) or 500 .mu.l in vitro translated [35S]-methionine labeled **human p53** in CLB-buffer and 25 .mu.l proteinA sepharose (stock solution: 50% w/w proteinA sepharose, Pharmacia in 50 mM Tris pH 7.4, 5 mM EDTA, 0.5% NP-40, 150 mM NaCl). The proteinA sepharose beads were washed three times in SNNTE (50 mM Tris pH 7.4, 5 mM EDTA, 5% sucrose, 1% NP-40, 0.5 M NaCl) and analyzed by 8% SDS-PAGE (Laemmli (1970) Nature 27, 690-). After completion of the run the gel was fixed in methanol/acetic acid for 30 min and washed twice 10 min in dH.sub.20 and once in 1 M sodium salicylate for 1/2 hour.

Detail Description Paragraph - DETX (57):

[0074] (10)3 cells are spontaneously immortalized fibroblasts which were derived from Balb c/J mice (Harvey, (1991) Genes Dev. 5, 2375-2385). The cells are devoid of endogenous p53 expression. Upon expression of **human or mouse mutant p53** protein the cells acquire the ability to form tumors in immunodeficient Balb c/J nu/nu mice (Dittmer, et al. (1993) Nature Genetics 4, 42-46). However, the majority of cell lines expressing mutant p53 alleles do not form tumors in immune-competent syngeneic Balb c/J mice (Table 1). The cell lines designated (10)3-248.1, (10)3-175.1 and (110).sub.3-273.1 are exceptions because they form tumors when injected subcutaneously (s.c.) into Balb c/J mice at 1*10.sup.7 cells per animal. All cell lines were generated by transfection of plasmids which contain in cis the gene for the respective **human mutant p53 under control of the human** cytomegalovirus (CMV) promoter/enhancer and the gene coding for resistance to G418 (Dittmer, et al. (1993) Nature Genetics 4, 42-46). (10)3-175.1 and (10)3-273.1 cell lines express high levels of **human p53** protein mutated at amino acid 175 and at amino acid 273 respectively. However, the growth characteristics of (10)3-175.1 and (10)3-273.1 in immunocompetent Balb c/J mice are quite different: (10)3-175.1 induced tumors grew progressively following a lag period of 20 days, whereas (10)3-273.1 induced tumors appeared as early as (10)3-175.1 induced tumors, but these tumors disappeared and only much later did a subset ([fraction (4/10)]) of the mice develop tumors (FIG. 1). Both (10)3-175.1 and (10)3-273.1 induced tumors exhibited undifferentiated neoplastic morphology typically seen in tumors induced by s.c. injection of tumor cells. The tumor cells continue to express **human p53** as demonstrated by immunohistochemistry of tumor sections using a **human p53**-specific antibody (mab1801) and immunoblot analysis. Cell lines established from these tumors continued to express **human p53** as demonstrated by immunoprecipitation of metabolically labeled cell extract using mab 1801 and were resistant to G418. This behavior is consistent with the notion that tumorigenicity of (10)3 cells is dependent upon continuous expression of the mutant p53 protein.

Detail Description Paragraph - DETX (62):

[0077] Remission of (10)3-273.1 induced tumors coincided with the appearance

of p53-specific antibodies. In contrast mice which had been injected with the parental (10)3 cells, (10)3-175.1 cells or untreated animals were devoid of anti-p53 antibodies (FIG. 2). The antibody response was measured by ELISA and immunoprecipitation using 3 .mu.l of whole mouse serum to precipitate **human wild-type p53 from SV40 transformed human** SV80 cell extracts when bound to protein A sepharose beads (FIG. 2). These experiments demonstrate that (10)3-273.1 cells are inherently immunogenic whereas (10)3-175.1 cells which also express p53 are not. These cell line specific differences were exploited to study a possible involvement of p53 as one of the TSRAs.

Detail Description Paragraph - DETX (68):

[0079] In order to address the question whether the protective effect seen after immunization with (10)3-273.1 cells was directed against mutant p53 or mutant p53 dependent TSRAs, (10)3-273.1 immunized animals were challenged with a tumorigenic clone of (10)3 cells (10)3-tx4BT87 which does not express p53. (10)3-tx4BT87 is a cell line derived from a tumor induced by spontaneously transformed (10)3 cells in Balb c/J mice. It forms progressive tumors in Balb c/J mice (FIG. 5A). Immunization with (10)3-273.1 cells delayed tumor onset but protected only a subset of animals ([fraction (3/7)]) (FIG. 5B). Immunization with (10)3-273.1 cells did not protect against a p53 negative cell line (10)3-tx4BT87 as effectively as against the mutant p53 positive (10)3-273.1NT24 cells. As seen after injection of live (10)3-273.1 cells (FIG. 2), the mitomycin C treated (10)3-273.1 cells were also able to elicit an anti-p53 IgG response. This was measured by ELISA and the ability of pooled serum samples taken at the time of tumor challenge to precipitate in vitro translated **human p53**. No anti-p53 antibodies could be detected in mice which were either non-immunized or mice immunized with (10)3 or (10)3-273.1 NT24 cells (FIG. 6). The difference in protection efficiency in experiment #2 (Table 2) of (10)3-273.1 NT24 versus (10)3-273.1 can be reconciled in light of the fact that (10)3-273.1 cells are much more effective in evoking an immune response than the (10)3-273.1 NT24 subclone. Both cells express similar levels of H2-K.sup.d (Table 3 and FIG. 7).

Detail Description Paragraph - DETX (73):

Susceptibility of Transgenic Mice Expressing **Human Mutant p53** to Mutant p53-Expressing Tumors

Detail Description Paragraph - DETX (74):

[0081] To further establish p53 as the dominant TSRA in (10)3-273.1 cells, we studied the susceptibility to (10)3-273.1 induced tumors in mice expressing **human p53** transgenes. Male mice which were heterozygous for **human p53** mutant 175 or 273 transgene were crossed with Balb c/J females and their offspring was injected s.c. with 1*10.sup.6 (10)3-273.1 cells per animal. Mice carrying the p53 transgene developed progressive growing tumors whereas their non transgenic littermates did not develop tumors or exhibited a delayed tumor onset (FIG. 9). Cell lines established from these tumors expressed **human p53** protein. The observed differences in tumor onset were significant to psO.OOl in a stratified Wilcoxon-Test. The differences are less pronounced compared to the challenge experiments in inbred Balb c/J mice which might be due to increased individual genetic variation between these DBAxC57Bl6xBalbc/J animals. The increased

tumor susceptibility of the transgenic mice demonstrates that the **p53 transgenic mice are tolerant to human p53** and thus are impaired in their ability to mount an effective immune response to tumor cells which express the **human mutant p53** whereas their siblings react similarly to normal Balb c/J mice.

Detail Description Paragraph - DETX (77):

[0082] The following table (Table 4) shows results concerning the ability to protect mice from tumors by immunizing them with **human p53** protein expressed in BCG bacteria. Commercially available Pasteur and Connaught (Ontario, Canada) BCG strains were used. The p53 gene is inserted into the BCG bacteria by known methods (see Snapper, S. B. et al., Proc. Natl. Acad. Sci. USA 85:6987-6991 (1988); Stover, C. K. et al., Vaccines 91, Cold Spring Harbor Laboratory Press, pp. 393-398 (1991); Stover, C. K. et al., Nature 351:456-460 (1991), Kalpana, B. V. et al., Proc. Natl. Acad. Sci. USA 88:5433-5437 (1991); and published International Applications Nos. WO 8806626, Sep. 9, 1988; WO 9000594. Jan. 25, 1990; and WO 9222326. Dec. 23, 1992).

Detail Description Paragraph - DETX (78):

[0083] As is shown in Table 4, the Connaught strain of BCG expressing **human p53** exons 5-11 at high level will protect against isogenic tumorigenic cells (see experiments #1 a, #1 b, and #1c, in which only two out of fifteen mice formed tumors). The BCG bactena alone, i.e., containing a vector but without p53 (experiment #2a) or not injecting BCG bacteria at all into the mice (experiments #2b, #2c) did not protect mice (21/25 mice formed tumors). The Pasteur strain expressing full length p53 at low levels was less effective than the Connaught strain expressing truncated p53 in protecting mice from the challenge as shown in experiments #3a, #3b, #3c (19/20 mice formed tumors, some of which progressed more slowly than the tumors formed in the mice in the control experiments (#2a-c)).

Detail Description Paragraph - DETX (84):

[0086] The (10)3-273.1NT24 cell line was derived from a nude mouse tumor induced by (10)3-273.1 cells (Dittmer, et al. (1993) Nature Genetics 4, 42-46). The cells express high levels of the **human 273 allele of p53** and of the MHC-I allele H2-K.sup.d. The cells were maintained in DMEM/10% FCS supplemented with 500-9/ml G418 and incubated at 37.degree. C. and 5% CO.sub.2.

Detail Description Paragraph - DETX (85):

[0087] Recombinant plasmids were generated using standard molecular biology techniques (Perbal. (1991) Methods in Molecular Biology (New York: Wiley). The pCMV-SN.sub.3 vector expressing the **human p53** cDNA is described in (Hinds, et al. (1990) Cell Growth Dift. 1, 571-580). The PMV261 and pMV262 expression plasmids are described in Stover, et al. (1993) J. Exp. Med. 178, 197-209.

Detail Description Paragraph - DETX (86):

[0088] Immunoblot analysis was carried out with extracts from BCG bacteria. The bacteria were lysed for 10 minutes on ice in lysis-buffer (10 mM Tris pH

7.4, 250 mM sucrose, 160 mM KCl, 50 mM .epsilon.amino-caproic acid, 0.5% NP-40 supplemented to 3 mM .beta.-mercaptoethanol, 1 mM PMSF and 0.28 TIU/ml aprotinin immediately prior to use) and sonicated. The insoluble particles were precipitated by spinning 20 min at maximum speed in an Eppendorf centrifuge. The protein concentration in the supernatant was determined by Biorad-assay (Biorad) and analyzed by 10% SDS-PAGE (Laemmli, (1970) Cancer Res. 53, 3468-a471). The proteins were then transferred to nitrocellulose (Amersham) by electroblotting for four hours in transfer buffer (25 mM Tris, 150 mM glycine) at <1.5 A. The membrane was stained with Ponceau S and the gel with Coomassie blue to check the transfer efficiency. The membrane was blocked for one hour in PBS/0.5% Tween 20/1% BSA. The primary antibody was diluted in PBS/0.5% Tween-20 and incubated at 4.degree. C. for two hours. The p53-specific monoclonal antibody pab421, which is specific for a C-terminal epitope of p53 (Harlow, et al. (1981) J. Virology 39, 861-869), or mabl801 which is specific for **human p53** (Banks, et al. (1986) J. Biochem. 159, 529-534), were used. After three washes 10 min in PBS/0.5% Tween-20 the membrane was incubated with peroxidase conjugated anti-mouse IgG (1:5000; Cappel) for 30 min at 4.degree. C. The membrane was washed three times with PBS/0.5% Tween-20/0.1% Triton X-100 and the antibody complex was visualized using the ECL system (Amersham).

Detail Description Paragraph - DETX (91):

[0091] The **human cDNA for wild-type p53 or the human** 175 mutant allele was cloned in the BCG expression vector pMV261. The cloning was confirmed by restriction digest. Wild-type and mutant cDNA PvuII-EcoRI **fragments from p53** vector (Hinds, et al., 1990) containing exons 5-11 (truncated) were fused to an eight amino acid leader of hsp60. The plasmids were amplified in E. coli and transformed into the Connaught strain of BCG. Expression was confirmed by immunoblot-analysis using the **human p53**-specific antibody pabl801 (FIG. 10).

Detail Description Paragraph - DETX (92):

[0092] The BamHI **fragment** of SN.sub.3 (Hinds, et al. (1990) Cell Growth Diff. 1, 571-580) containing the full-length **p53** ORF was cloned into three different frames behind the BCG hsp60 promoter. This introduced a leader of 143 nucleotides between the promoter and the N-**terminus of p53,** which was subsequently found to contain a short ORF. This construct was also used to transform the Pasteur strain of BCG. The clone (BCG-1SN.sub.3) expressed full-length **p53** protein (FIG. 10). The expression level of full-length **p53** protein was low compared to the expression level of the C-**terminal fragments**. C-**terminal** or N-**terminal** GST-**fragments** were expressed at higher level than full-length GST-**p53** protein. Immunoprecipitation of bacterial extract using either pabl801 directed against an N-**terminal** epitope of **p53** or pab421 directed against a C-**terminal** epitope of **p53** confirmed that full-length **p53** was produced by BCG-ISN.sub.3 bacteria. The BCG hsp60 promoter was constitutively active and produced high levels of recombinant **p53** protein even prior to heat shock (FIG. 11).

Detail Description Paragraph - DETX (101):

[0097] 5 mice each were injected s.c. with 5*10.sup.7 avipoxvirus genus

canary poxvirus (ALVAC) particles, boosted 28 days after immunization and injected s.c. with 10.sup.5 (10)3-273.1 NT24 cells 52 days after immunization. Tumorigenicity was analyzed as before (FIG. 14). Mice immunized with the vector alone developed tumors at 35.+-.21 days. Mice immunized with ALVAC expressing murine wild-type p53 or the murine 135 allele of pS3 developed tumors later (70.+-.50 and 55.+-.50 days, respectively). Mice immunized with ALVAC expressing **human wild-type p53, the human mutant p53 allele 175 or the human mutant p53** allele 273 were protected against (10)3-273.1 NT24 induced tumors (p.ltoreq.0.001 for n=15 mice using the Mann-Whitney U Test). Only 1 mouse out of 5 immunized with ALVAC expressing either the 175 or 273 allele of p53 developed a tumor. None of the mice immunized with ALVAC expressing the wild-type **human p53** allele developed a tumor.

Claims Text - CLTX (51):

51. A vaccine composition according to claim 1 wherein the mutant **p53 protein is a fragment expressed by a truncated mutant p53** gene.

Claims Text - CLTX (54):

54. A method according to claim 13 wherein the mutant **p53 protein is a fragment expressed by a truncated mutant p53** gene.

Claims Text - CLTX (57):

57. A recombinant antigen presenting cell according to claim 25 wherein the mutant **p53 protein is a fragment expressed by a truncated mutant p53** gene.

Claims Text - CLTX (60):

60. A vaccine composition according to claim 26 wherein the wild-type **p53 protein is a fragment expressed by a truncated wild-type p53** gene.

Claims Text - CLTX (63):

63. A method according to claim 38 wherein the wild-type **p53 protein is a fragment expressed by a truncated wild-type p53** gene.

Claims Text - CLTX (66):

66. A recombinant antigen presenting cell according to claim 50 wherein the wild-type **p53 protein is a fragment expressed by a truncated wild-type p53** gene.

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ABSTRACT:

The present invention is directed towards the diagnosis of malignant cancer by detection of the mts-1 MRNA or the mts-1 protein, encoded by the mts-1 gene. The present invention contemplates the use of recombinant mts-1 DNA and

antibodie4 directed against the mts-1 protein to diagnose the metastatic potential of several types of tumor cells, including, for example, thyroid, epithelial, lung, liver and kidney tumor cells. The present invention is also directed to mammalian cell lines and tumors with high and low metastatic potential which have been developed to serve as useful model systems for in vitro and in vivo anti-metastasis drug screening.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This is a Continuation-In-Part of U.S. Ser. No. 08/468,942 filed Jun. 6, 1995, which is a Divisional of U.S. Ser. No. 190,560 filed Jan. 31, 1994, which is a Continuation-In-Part of U.S. Ser. No. 981,455 filed Nov. 25, 1992, which is a Rule 60 Continuation of U.S. Ser. No. 550,600 filed Jul. 9, 1990.

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Summary of Invention Paragraph - BSTX (12):

[0011] The nucleotide and amino acid sequences of **human p53** have been reported by Zakut-Houri et al, EMBO J. 4: 1251-1255, 1985). The ability of p53 to bind DNA in a sequence-specific manner maps to amino acid residues 90-290 of **human p53** (Pavletich et al, Genes Dev. 7: 2556-2564, 1993; and Wang et al, Genes Dev. 7: 2575-2586 1993); the tetramerization domain maps to amino acid residues 322-355 of **human p53**. The DNA binding- regulation domain maps to amino acid residues 364-393 of **human p53** or to the corresponding region encompassing residues 361-390 of mouse p53 (Hupp et al., Cell 71: 875-886, 1992; and Halazonetis et al., EMBO J. 12: 1021-1028, 1993).

Summary of Invention Paragraph - BSTX (13):

[0012] Inactivation of **p53 is associated with more than half of all human** tumors. The inactivation can occur by mutation of the p53 gene or through binding of p53 to viral or cellular oncogene proteins, such as the SV40 large T antigen and MDM2. Mutations of the **p53 protein in most human** tumors involve the sequence-specific DNA binding domain (Bargonetti et al., Genes Dev. 6: 1886-1898, 1992).

Summary of Invention Paragraph - BSTX (24):

[0022] Another embodiment of the present invention provides a method for intercepting the binding between **p53** and Mts-1 in a subject by administering to the subject, an effective amount of a **peptide which prevents the interaction between p53** and Mts-1 by binding to Mts-1. For example, one such **peptide comprises the C-terminal region of p53** (amino acid 289-393 of **human p53** or amino acid 289-390 of murine **p53**), in particular, amino acid 360-393 of **human p53** or amino acid 360-390 of murine **p53**. Functional **fragments** or analogs of such **peptides** are also within the scope of the present invention. Another example of a binding-intercepting **peptide** comprises amino acid 1909-1937 of non-muscle myosin heavy chain or functional **fragments** of analogs thereof.

Summary of Invention Paragraph - BSTX (25):

[0023] In one embodiment, the present invention provides methods of treating a tumor in a subject by administering to the subject, a therapeutically effective amount of a nucleic acid molecule coding for a **peptide which prevents the binding of Mts-1 to p53**.

Summary of Invention Paragraph - BSTX (26):

[0024] In one embodiment, the present invention provides methods of treating a tumor in a subject by administering to the subject, a therapeutically effective amount of a **peptide which prevents the binding of Mts-1 to p53**.

Brief Description of Drawings Paragraph - DRTX (34):

[0060] FIG. 21 depicts Mts1 interaction with target proteins in a blot-overlay assay. Recombinant full size **p53** (1), N-**terminal** domain (2), DNA-binding domain (3), C-**terminal** domain (4) and the **fragment** of the non-muscle myosin (5) after gel electrophoresis were transferred onto nitrocellulose membrane. Identical membranes were incubated with different batches of the recombinant Mts1 protein (Mts1-a, Mts1-b, Mts1-c and Mts1-d). Mts1 bound to the fixed proteins was detected by the anti-Mts1 serum. The graph at the upper left depicts the schematic localization of the proteins on the membranes.

Detail Description Paragraph - DETX (74):

[0141] The present invention provides **peptides which prevent p53 from binding to Mts1, e.g., a peptide comprising aa 289-393 of human p53, a peptide comprising aa 360-393 of human p53, a peptide comprising aa 289-390 of murine p53, a peptide comprising aa 360-390 of murine p53, a peptide** comprising the C-**terminal** nonmuscle myosin heavy chain, a **peptide** comprising amino acid 1909-1937 of human nonmuscle myosin heavy chain. Functional **fragments** and analogs of these **peptides** are also contemplated by the present invention.

Detail Description Paragraph - DETX (75):

[0142] "Functional fragments" refer to peptide fragments that have the same function as the **peptide in issue, namely, the function of interfering the Mts1-p53** interaction by binding to Mts-1.

Detail Description Paragraph - DETX (76):

[0143] By "analogs" it means variants of a peptide in issue. The variations include substitutions, insertions or deletions of one or more amino acid residues, or modifications of the side chains of the amino acid residues. Thus, analogs of a peptide can include homologous peptides from other mammalian species, peptides containing non-natural amino acid residues, peptides having chemical modifications on the side groups of amino acid residues, as well as peptides artificially designed to resemble the three dimensional structure of the binding site on **human p53**.

Detail Description Paragraph - DETX (77):

[0144] A variety of techniques are available to those skilled in the art to make various **fragments or anal gs f p53**. Such techniques include standard chemical synthesis, recombinant expression, and structural modeling (also called `mimetics`). The sequences of p53 from a number of mammalian species are highly conserved and are available to those skilled in the art, e.g.; via Databases such as Genank.

Detail Description Paragraph - DETX (82):

[0149] The present invention also contemplates pharmaceutical compositions which include, as an active ingredient, an Mts1**-p53 binding intercepting peptide** as described hereinabove, and a pharmaceutically acceptable carrier.

Detail Description Paragraph - DETX (83):

[0150] In another aspect of the present invention, Mts1**-p53 binding-intercepting peptides** or nucleic acid molecules encoding thereof are used for treating tumors.

Detail Description Paragraph - DETX (88):

[0155] In one embodiment, the present invention provides methods of treating a tumor in a subject by administering to the subject, a therapeutically effective amount of a **peptide which prevents the binding of Mts-1 to p53**. Preferred binding intercepting peptides have been described hereinabove.

Detail Description Paragraph - DETX (89):

[0156] In another embodiment, the present invention provides methods of treating a tumor in a subject by administering to the subject, a therapeutically effective amount of a nucleic acid molecule coding for a **peptide which prevents the binding of Mts-1 to p53**.

Detail Description Paragraph - DETX (269):

[0299] The following mouse wild type p53 PCRs were designed: #1-full size coding region (390 aa) was amplified using primers: forward CGGGATCCGACTGGATGACTGCCATGGA (SEQ ID NO:10) (having a BamHI site), reverse CGAAGCTTCAGTCTGAGTCAGGCCCCACT (SEQ ID NO:11) (including a HindIII site); #2-N-terminal domain (106 aa): forward, same as the forward primer for #1, and reverse CGAAGTCTTGAAGCCATAGTTGCCCTGGTAAG (SEQ ID NO:12) (including a HindIII site); #3-DNA-binding domain (185 aa): forward CGGGATCCCACCTGGGCTTCCTGCATGCT (SEQ ID NO:13) (including a BamHI site), reverse CGAAGCTTGGACTTCCTTTTTTGCGGAAATTTTC (SEQ ID NO:14) (including a HindIII site); #4-C-terminal (99 aa): forward CGGGATCCCTTTGCCCTGAACTGCCCC- CA (SEQ ID NO:15) (including a BamHI site), and reverse--same as the reverse primer for #1. The PCR products were digested with BamHI/HindIII and cloned in eukaryotic expression vector pxmyctag, containing a CMV promoter and 8-aa myc tag, and bacterial expression vector pQE30 (Qiagen). PSP65m65 plasmid DNA was used for the amplification of p53. Human pC53-SN3 **(human wild type p53) and pC53-SCX3**

(human mutant Human mutant p53-pC53-SCX3 (143.sup.Val-Ala) eukaryotic expression plasmids were obtained. For conditional expression, mts1 cDNA was excised, cloned in pUHD 10-3 and used for transfection of cell lines producing reverse tetracycline-controlled transactivator (pUHD172-neo) (Clontech).

Detail Description Paragraph - DETX (280):

[0310] For in vitro pull down assay, 1 .mu.g recombinant Mts1 was mixed with recombinant full size **p53 and its domain peptides** in 150 mM NaCl-50 mM Tris-HCl pH 8.0-0.5% NP-40 and precleaned on Protein A-Sepharose on the presence of protease inhibitors at 1 hour in cold room. To the precleaned mixtures, fresh **portions of the protein A-sepharose and the corresponding anti-p53** antibodies were added: pAb421 for full-size and C**-terminal** domain, pAb240 for DNA-binding core domain and E-19 for the N**-terminal** domain, and incubated for 2 hours in the cold room. Following 5 washes, immunoprecipitates were denatured by heating at 100.degree. C.-5 min, separated in 15% PAAG and-transferred to Immobilon-P (Millipore). To detect the co-immunoprecipitated Mts1 protein, membranes were probed with anti-Mts1 antibody and developed by the ECL System. Recombinant **human wild type GST-p53** and GST-**p53**-.DELTA.30 (deletion mutant lacking amino acid residues 364-393) fusion proteins were used for pull down experiments with the Mts1 recombinant protein. 5.mu.g of GST and GST-fusion proteins coupled with Glutathione-sepharose beads were incubated with 2 .mu.g of the Mts1 protein in NP-40 buffer (1% NP-40, 50 mM Tris-HCl pH 8.0-150 mM NaCl) for 2 h in the cold room with rotation. Beads with proteins bound were washed 5 times with NP-40-buffer. Proteins were isolated by boiling in the protein loading buffer for 5 min and analyzed using Western blotting.

Detail Description Paragraph - DETX (282):

[0312] Reactions were performed in a mixture (2 .mu.l) containing 50 mM Tris-HCl pH 7.6, 0.2 M NaCl, 10 mM MgCl.sub.2, 4 mM CaCl.sub.2, 2 mM dithiothreitol, 151 .mu.l ATP (Amersham Pharmacia Biotech), 25 .mu.Ci [.gamma.-.sup.32P]-ATP (5000 Ci/nnol, Amersham Pharmacia Biotech), 1 .mu.M recombinant wild type **p53 or this protein fragments** for 30 min at 30.degree. C. PKC assay was done in the presence of 7.5 .mu.g of phosphatidylserine (Sigma) by 0.025 .mu.g PKC (Roche). CKII was purchased from New England BioLabs Inc., and 50 units were applied per each reaction. Recombinant Mts1 was sued in concentrations of 3, 5 and 9 .mu.M reactions were **terminated** by 15% SDS-PAGE. Gels were fixed in 10% trichloracetic acid, dried and exposed to Kodak x-ray film.

Detail Description Paragraph - DETX (294):

[0322] Another approach, Far-Western blot analysis, was also employed to assess the interaction between Mts1 and **p53**. Full size **p53** and its functional domains, expressed in E.coli, were separated on SDS-PAGE and transferred into Immobilon-P. Filters were incubated with recombinant Mts1 in conditions allowing the interaction with the proteins fixed on the membrane. Mts1 bound to **p53** proteins on the filter, was detected with anti-Mts1 antibody. Data shown in FIG. 21, consistent with the IP results, indicated that Mts1 was able to bind full-size **p53** and its C**-terminal** domain. As a positive control we have used recombinant **fragment** of non-muscle myosin which is known as a target for

Mts1 protein (FIG. 21, lane 5). BSA loaded in 5x excess did not reveal nonspecific mts1 binding in Far-Western assay, neither did N-**terminal** or DNA-binding domains.

Detail Description Paragraph - DETX (298):

[0324] As shown in FIG. 22, Mts-1 inhibited the **phosphorylation** of full-size **p53 and the C-terminal protein fragment** by PKC. Addition of the same concentrations of Mts1 to the PKC reaction mixture did not affect the **phosphorylation** of the N-**terminal** and DNA-binding domains of **p53**. No interference of Mts1 was shown with CK II **phosphorylation of p53** and its domains (FIG. 23). These-observations indicate that Mts1 specifically inhibited the **phosphorylation** of PKC of the C-**terminal** domain of **p53**.

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INVENTOR-INFORMATION:

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US-CL-CURRENT: 514/2, 514/44

ABSTRACT:

The present invention is directed towards the diagnosis of malignant cancer by detection of the mts-1 MRNA or the mts-1 protein, encoded by the mts-1 gene. The present invention contemplates the use of recombinant mts-1 DNA and antibodie4 directed against the mts-1 protein to diagnose the metastatic potential of several types of tumor cells, including, for example, thyroid, epithelial, lung, liver and kidney tumor cells. The present invention is also directed to mammalian cell lines and tumors with high and low metastatic

potential which have been developed to serve as tseful model systems for in vitro and in vivo anti-metastasis drug screening.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This is a Continuation-In-Part of U.S. Ser. No. 08/468,942 filed Jun. 6, 1995, which is a Divisional of U.S. Ser. No. 190,560 filed Jan. 31, 1994, which is a Continuation-In-Part of U.S. Ser. No. 981,455 filed Nov. 25, 1992, which is a Rule 60 Continuation of U.S. Ser. No. 550,600 filed Jul. 9, 1990.

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Summary of Invention Paragraph - BSTX (12):

[0011] The nucleotide and amino acid sequences of **human p53** have been reported by Zakut-Houri et al, EMBO J. 4: 1251-1255, 1985). The ability of p53 to bind DNA in a sequence-specific manner maps to amino acid residues 90-290 of **human p53** (Pavletich et al, Genes Dev. 7: 2556-2564, 1993; and Wang et al, Genes Dev. 7: 2575-2586 1993); the tetramerization domain maps to amino acid residues 322-355 of **human p53**. The DNA binding-regulation domain maps to amino acid residues 364-393 of **human p53** or to the corresponding region encompassing residues 361-390 of mouse p53 (Hupp et al., Cell 71: 875-886, 1992; and Halazonetis et al., EMBO J. 12: 1021-1028, 1993).

Summary of Invention Paragraph - BSTX (13):

[0012] Inactivation of **p53 is associated with more than half of all human** tumors. The inactivation can occur by mutation of the p53 gene or through binding of p53 to viral or cellular oncogene proteins, such as the SV40 large T antigen and MDM2. Mutations of the **p53 protein in most human** tumors involve the sequence-specific DNA binding domain (Bargonetti et al., Genes Dev. 6: 1886-1898, 1992).

Summary of Invention Paragraph - BSTX (24):

[0022] Another embodiment of the present invention provides a method for intercepting the binding between **p53** and Mts-1 in a subject by administering to the subject, an effective amount of a **peptide which prevents the interaction between p53** and Mts-1 by binding to Mts-1. For example, one such **peptide comprises the C-terminal region of p53** (amino acid 289-393 of **human p53** or amino acid 289-390 of murine **p53**), in particular, amino acid 360-393 of **human p53** or amino acid 360-390 of murine **p53**. Functional **fragments** or analogs of such **peptides** are also within the scope of the present invention. Another example of a binding-intercepting **peptide** comprises amino acid 1909-1937 of non-muscle myosin heavy chain or functional **fragments** of analogs thereof.

Summary of Invention Paragraph - BSTX (25):

[0023] In one embodiment, the present invention provides methods of treating a tumor in a subject by administering to the subject, a therapeutically

effective amount of a nucleic acid molecule coding for a **peptide which prevents the binding of Mts-1 t p53**.

Summary of Invention Paragraph - BSTX (26):

[0024] In one embodiment, the present invention provides methods of treating a tumor in a subject by administering to the subject, a therapeutically effective amount of a **peptide which prevents the binding of Mts-1 to p53**.

Brief Description of Drawings Paragraph - DRTX (34):

[0060] FIG. 21 depicts Mts1 interaction with target proteins in a blot-overlay assay. Recombinant full size **p53** (1), N-**terminal** domain (2), DNA-binding domain (3), C-**terminal** domain (4) and the **fragment** of the non-muscle myosin (5) after gel electrophoresis were transferred onto nitrocellulose membrane. Identical membranes were incubated with different batches of the recombinant Mts1 protein (Mts1-a, Mts1-b, Mts1-c and Mts1-d). Mts1 bound to the fixed proteins was detected by the anti-Mts1 serum. The graph at the upper left depicts the schematic localization of the proteins on the membranes.

Detail Description Paragraph - DETX (74):

[0141] The present invention provides **peptides which prevent p53 from binding to Mts1, e.g., a peptide comprising aa 289-393 of human p53, a peptide comprising aa 360-393 of human p53, a peptide comprising aa 289-390 of murine p53, a peptide comprising aa 360-390 of murine p53, a peptide** comprising the C-**terminal** nonmuscle myosin heavy chain, a **peptide** comprising amino acid 1909-1937 of human nonmuscle myosin heavy chain. Functional **fragments** and analogs of these **peptides** are also contemplated by the present invention.

Detail Description Paragraph - DETX (75):

[0142] "Functional fragments" refer to peptide fragments that have the same function as the **peptide in issue, namely, the function of interfering the Mts1-p53** interaction by binding to Mts-1.

Detail Description Paragraph - DETX (76):

[0143] By "analogs" it means variants of a peptide in issue. The variations include substitutions, insertions or deletions of one or more amino acid residues, or modifications of the side chains of the amino acid residues. Thus, analogs of a peptide can include homologous peptides from other mammalian species, peptides containing non-natural amino acid residues, peptides having chemical modifications on the side groups of amino acid residues, as well as peptides artificially designed to resemble the three dimensional structure of the binding site on **human p53**.

Detail Description Paragraph - DETX (77):

[0144] A variety of techniques are available to those skilled in the art to make various **fragments or anal gs of p53**. Such techniques include standard

chemical synthesis, recombinant expression, and structural modeling (also called `mimetics`). The sequences of p53 from a number of mammalian species are highly conserved and are available to those skilled in the art, e.g., via Databases such as GenBank.

Detail Description Paragraph - DETX (82):

[0149] The present invention also contemplates pharmaceutical compositions which include, as an active ingredient, an Mts1-**p53 binding intercepting peptide** as described hereinabove, and a pharmaceutically acceptable carrier.

Detail Description Paragraph - DETX (83):

[0150] In another aspect of the present invention, Mts1-**p53 binding-intercepting peptides** or nucleic acid molecules encoding thereof are used for treating tumors.

Detail Description Paragraph - DETX (88):

[0155] In one embodiment, the present invention provides methods of treating a tumor in a subject by administering to the subject, a therapeutically effective amount of a **peptide which prevents- the binding of Mts-1 to p53**. Preferred binding intercepting peptides have been described hereinabove.

Detail Description Paragraph - DETX (89):

[0156] In another embodiment, the present invention provides methods of treating a tumor in a subject by administering to the subject, a therapeutically effective amount of a nucleic acid molecule coding for a **peptide which prevents the binding of Mts-1 to p53**.

Detail Description Paragraph - DETX (267):

[0297] The following mouse wild type p53 PCRs were designed: #1--full size coding region (390 aa) was amplified using primers: forward CGGGATCCGACTGGATGACTGCCATGGA (SEQ ID NO:l0) (having a BamHI site), reverse CGAAGCTTCAGTCTGAGTCAGGCCCCACT (SEQ ID NO:11) (including a HindIII site); #2--N-terminal domain (106 aa): forward, same as the forward primer for #1, and reverse CGAAGTCTTGAAGCCATAGTTGCCCTGGTAAG (SEQ ID NO:12)(including a HindIII site); #3--DNA-binding domain (185 aa) : forward CGGGATCCCACCTGGGCTTCCTGCATGCT (SEQ ID NO:13) (including a BamHI site), reverse CGAAGCTTGGACTTCCTTTTTTGCGGAAATTTTC (SEQ ID NO: 14) (including a HindIII site); #4--C-terminal (99 aa): forward CGGGATCCCTTTGCCCTGAACTGCCCCCA (SEQ ID NO:15) (including a BamHI site), and reverse--same as the reverse primer for #1. The PCR products were digested with BamHI/HindIII and cloned in eukaryotic expression vector pXmyctag, containing a CMV promoter and 8-aa myc tag, and bacterial expression vector pQE30 (Qiagen). PSP65m65 plasmid DNA was used for the amplification of p53. Human pC53-SN3 **(human wild type p53) and pC53-SCX3 (human mutant Human mutant p53**-pC53-SCX3 (.sub.143.sup.Val.fwdarw.Ala) eukaryotic expression plasmids were obtained. For conditional expression, mts1 cDNA was excised, cloned in pUHD 10-3 and used for transfection of cell lines producing reverse tetracycline-controlled transactivator (pUHD172-neo) (Clontech).

Detail Description Paragraph - DETX (278):

[0308] For in vitro pull down assay, 1 .mu.g recombinant Mts1 was mixed with recombinant full size **p53 and its domain peptides** in 150 mM NaCl-50 mM Tris-HCl pH 8.0-0.5% NP-40 and precleaned on Protein A-Sepharose on the presence of protease inhibitors at 1 hour in cold room. To the precleaned mixtures, fresh **portions of the protein A-sepharose and the corresponding anti-p53** antibodies were added: pAb421 for full-size and C**-terminal** domain, pAb240 for DNA-binding core domain and E-19 for the N**-terminal** domain, and incubated for 2 hours in the cold room. Following 5 washes, immunoprecipitates were denatured by heating at 100.degree. C.-5 min, separated in 15% PAAG and transferred to Immobilon-P (Millipore). To detect the co-immunoprecipitated Mts1 protein, membranes were probed with anti-Mts1 antibody and developed by the ECL System. Recombinant **human wild type-GST-p53** and GST**-p53-**.DELTA.30 (deletion mutant lacking amino acid residues 364-393) fusion proteins were used for pull down experiments with the Mts1 recombinant protein. 5.mu.g of GST and GST-fusion proteins coupled with Glutathione-sepharose beads were incubated with 2 .mu.g of the Mts1 protein in NP-40 buffer (1% NP-40, 50 mM Tris-HCl pH 8.0-150 mM NaCl) for 2h in the cold room with rotation. Beads with proteins bound were washed 5 times with NP-40-buffer. Proteins were isolated by boiling in the protein loading buffer for 5 min and analyzed using Western blotting.

Detail Description Paragraph - DETX (280):

[0310] Reactions were performed in a mixture (2.mu.l) containing 50 mM Tris-HCl pH 7.6, 0.2 M NaCl, 10 mM MgCl.sub.2, 4mM CaCl.sub.2, 2 mM dithiothreitol, 15 .mu.l ATP (Amersham Pharmacia Biotech), 25 .mu.Ci [.gamma.-.sup.32P]-ATP (5000 Ci/nnol, Amersham Pharmacia Biotech), 1 .mu.M recombinant wild type **p53 or this protein fragments** for 30 min at 30.degree. C. PKC assay was done in the presence of 7.5 .mu.g of phosphatidylserine (Sigma) by 0.025 .mu.g PKC (Roche). CKII was purchased from New England BioLabs Inc., and 50 units were applied per each reaction. Recombinant Mts1 was sued in concentrations of 3,5 and 9 .mu.M reactions were **terminated** by 15% SDS-PAGE. Gels were fixed in 10% trichloracetic acid, dried and exposed to Kodak x-ray film.

Detail Description Paragraph - DETX (292):

[0320] Another approach, Far-Western blot analysis, was also employed to assess the interaction between Mts1 and **p53**. Full size **p53** and its functional domains, expressed in E.coli, were separated on SDS-PAGE and transferred into Immobilon-P. Filters were incubated with recombinant Mts1 in conditions allowing the interaction with the proteins fixed on the membrane. Mts1 bound to **p53** proteins on the filter, was detected with anti-Mts1 antibody. Data shown in FIG. 21, consistent with the IP results, indicated that Mts1 was able to bind full-size **p53** and its C**-terminal** domain. As a positive control we have used recombinant **fragment** of non-muscle myosin which is known as a target for Mts1 protein (FIG. 21, lane 5). BSA loaded in 5.times. excess did not reveal nonspecific mts1 binding in Far-Western assay, neither did N**-terminal** or DNA-binding domains.

Detail Description Paragraph - DETX (296):

[0322] As shown in FIG. 22, Mts-1 inhibited the **phosph rylation** of full-size **p53 and the C-terminal protein fragment** by PKC. Addition of the same concentrations of Mts1 to the PKC reaction mixture did not affect the **phosphorylation** of the N**-terminal** and DNA-binding domains of **p53**. No interference of Mts1 was shown with CKII **phosphorylation of p53** and its domains (FIG. 23). These observations indicate that Mts1 specifically inhibited the **phosphorylation** of PKC of the C**-terminal** domain of **p53**.

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ABSTRACT:

Specific sequences in the human genome are the sites of strong binding of wild-type p53 protein, but not mutant forms of the protein. These sequences are used diagnostically to detect cells in which the amount of wild-type p53 is

diminished. The sequences can also be used to screen for agents which correct for loss of wild-type p53 to DNA in cancer cells.

[0001] This application is a continuation-in-part of U.S. Ser. No. 07/715,182 filed Jun. 14, 1991.

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Summary of Invention Paragraph - BSTX (9):
   [0008] It is yet another object of the invention to provide a double-stranded DNA **fragment which contains a p53**-specific DNA binding site.

Summary of Invention Paragraph - BSTX (13):
   [0012] These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment a method is provided for detecting the presence of wild-type p53 protein in a cell, comprising the steps of: contacting a **p53-specific-binding DNA fragment** with a cell lysate from a tissue of a human to bind the DNA **fragment to wild-type p53** present in the cell lysate; and detecting the binding of the **p53-specific-binding DNA fragment to wild-type p53**.

Summary of Invention Paragraph - BSTX (15):
   [0014] In yet another embodiment a double-stranded DNA **fragment is provided which comprises a p53-specific-DNA binding site, wherein the fragment** comprises more than one monomer repeat of the sequence 5'-RRRCWWGYYY-3' and wherein the fragment is covalently attached to an insoluble polymeric support.

Summary of Invention Paragraph - BSTX (17):
   [0016] In yet another embodiment of the invention a method is provided for identifying compounds which specifically bind to p53-specific DNA binding sequences, comprising the steps of: contacting a **p53-specific DNA binding fragment** immobilized on a solid support with a test compound to bind the test compound to the DNA fragment; and determining the amount of. test compound which is bound to the DNA fragment.

Summary of Invention Paragraph - BSTX (18):
   [0017] In even another embodiment of the invention a method is provided for identifying compounds which specifically bind to p53-specific-DNA binding sequences, comprising the steps of: contacting a **p53-binding DNA fragment** immobilized on a solid support with both a test compound and wild-type p53 protein to bind the wild-type **p53 protein to the DNA fragment; determining the amount of wild-type p53 protein which is bound to the DNA fragment, inhibition of binding of wild-type p53** protein by the test compound suggesting binding of the test compound to the p53-specific DNA binding sequences.

Summary of Invention Paragraph - BSTX (23):

[0022] In another embodiment of the invention a method is provided of diagnosing tumor-inducing or hyperplastia-inducing strains of human papilloma virus (HPV) comprising: contacting cells or cell extracts of patients suspected of being infected by HPV with a **p53-specific binding DNA fragment; and detecting the amount of wild-type p53 in said cells or cell extract which binds to said DNA fragment, absence of bound p53** indicating infection by strains of HPV which sequester p53.

Brief Description of Drawings Paragraph - DRTX (2):

[0024] FIG. 1A. Screening for **fragments bound by p53** using an immunoprecipitation assay. Panel 1 contains the hFosAva2 clone; panel 2, 772 C.sub.BE; panel 3, Lambda 5R; panel 4, a pool of clones with inserts of randomly cloned human genomic sequences. 772 C.sub.BE and Lambda SR contain Hinfl **fragments (259 and 190 bp, respectively) which bound p53** relatively strongly (arrowheads). "C"--control lane, containing 2% of the labelled DNA used in the binding reactions. "B"--bound DNA recovered from the immunoprecipitate.

Brief Description of Drawings Paragraph - DRTX (4):

[0026] FIG. 2. Relative abilities of wild-type and mutant **p53 to precipitate fragment** A. "C"--control lanes, containing 2% of the labelled DNA used in the binding reaction, "B"--bound DNA recovered from the immunoprecipitate.

Brief Description of Drawings Paragraph - DRTX (6):

[0028] FIG. 2B. Lysates from a vaccinia virus system (Vac) producing the wild-type (wt), mutant (175.sup.his), or no **p53 protein (-), were used to immunoprecipitate labelled C.sub.BE fragments**. Equivalent quantities of p53 were present in the wild-type and mutant p53 lysates, as assessed by Western blot. In the "Bac" lane, affinity-purified p53 produced in baculovirus-infected insect cells was used in place of the vaccinia-infected lysates.

Brief Description of Drawings Paragraph - DRTX (9):

[0031] FIG. 4. Binding of various subfragments of **fragments A and B to p53** from vaccinia-infected cell lysates.

Brief Description of Drawings Paragraph - DRTX (16):

[0038] FIG. 6B shows Southern blot analysis of transfected clonal lines. The exogenous **p53 gene was present on a 1.8 kb BamHI fragment**. The endogenous p53 gene gave rise to a 7.8 kb BamHI fragment. Other sized fragments presumably arose by rearrangements.

Brief Description of Drawings Paragraph - DRTX (19):

[0041] FIG. 8. Isolation of **human genomic sequences which bound t p53**.

Brief Description of Drawings Paragraph - DRTX (20):
   [0042] FIG. 8A. Experimental strategy used for isolation and analysis of human genomic DNA **fragments which b und to p53**.

Brief Description of Drawings Paragraph - DRTX (21):
   [0043] FIG. 8B. Immunoprecipitation (IP) assays of cloned fragments. Clones of amplified and selected (AS) DNA were tested for the presence of **p53-binding fragments** by IP. For each clone, the bound DNA is shown in the B lane, adjacent to a control (c) lane containing 2% of the total end-labeled DNA used in the binding assay. In this representative experiment, eight binding fragments were identified, representing six unique genomic fragments. The inserts from the clones in lanes labeled, 2, 3, 5, 9, 10, and 11 contained **p53-binding fragments,** while the other lanes contained none. The clones in lanes 2 and 5 each contained two binding fragments.

Brief Description of Drawings Paragraph - DRTX (26):
   [0048] FIG. 11B. Comparison of the ability of wild-type and mutant p53 to bind to the consensus dimer. In vitro translated p53 proteins were tested for the ability to bind the consensus dimer by IP. Two percent of the total DNA used for binding is shown in lane 1. Lane 7 shows binding to baculovirus-produced **human wild-type p53** protein. Lanes 2 to 6 show binding of in vitro translated wild-type and mutant p53 proteins. The mutant p53 proteins contained changes at codon 143 (val to ala), 175 (arg to his), 248 (arg to trp), and 273 (arg to his).

Brief Description of Drawings Paragraph - DRTX (31):
   [0053] FIG. 13A. Relative DNA-binding abilities of various length concatemers of a p53-binding sequence (PG.sub.n series), using an immunoprecipitation assay. Clones were cleaved by restriction endonucleases to extricate the concatemers, end-labelled, incubated with purified baculovirus-produced wild-type **human p53,** immunoprecipitated with anti-**p53 and protein A-Sepharose, and bound fragments** recovered and separated on a nondenaturing polyacrylamide gel. C, control lane, containing 2% of the labeled DNA used in the binding reactions. B, bound DNA recovered from the binding reactions.

Detail Description Paragraph - DETX (2):
   [0065] It is a finding of this invention that wild-type **p53 protein binds specific fragments** of human chromosomal DNA. Each of the fragments contains more than one monomer of the double-stranded motif 5'-RRRCWWGYYY-3' separated by 0 to 13 bp. Some of these sequences are found near origins of replication of certain animal viruses and animal cells. See Jelinek et al, Proc. Natl. Acad. Sci. USA, vol. 77, pp. 1398-1402 (1980). Four mutant forms of **p53 protein which are commonly found in human** tumors do not have the ability to bind to these sequences. Thus, a function of p53 may be mediated by its ability to bind to specific DNA sequences in the human genome.

Detail Description Paragraph - DETX (5):

[0068] It has been found that **p53 will specifically bind t   ther sequences in the human** genome with similar sequence motifs. Using a strategy coupling immunoprecipitation to "whole-genome PCR" (Kinzler, et al., Nucleic Acids Research, 17:3645-3653 (1989)), nineteen **human DNA fragments that bind to p53** have been identified. Each of the fragments contain a sequence which conforms to a dimer of the double-stranded motif 5'-RRRCWWGYYY-3', separated by 0 to 13 bp. These dimers directly mediate binding, as assessed by DNase I protection and methylation interference assays. The consensus dimers contain a striking symmetry, with four 5'-RRRCW-3' units oriented in alternating directions. A synthetic monomer containing the 10 bp consensus sequence is insufficient for binding, while the combination of two or more monomers bind strongly to wt p53, but negligibly to p53 mutants. Thus, more than one monomer appears to be required for binding. The spacing between monomers may be from 0 to 40 nucleotides, although all natural binding sites isolated have spacings of less than 15 nucleotides. The symmetry of the four half-sites within the consensus dimers suggests that p53 interacts with DNA as a tetrameric protein. The eighteen unique clones shown in FIG. 10 allow the identification of adjacent genes which may be regulated by p53 and may mediate its growth-suppressive action.

Detail Description Paragraph - DETX (8):

[0071] Based on the sequence information of the **p53 specific-DNA-binding fragments,** a number of diagnostic and therapeutic methods have been devised. According to one such method, cell lysates are tested for the presence or absence of wild-type p53 by virtue of its specific DNA binding ability. As it is known for various cancers and stages of cancers that one or both of the p53 alleles in tumor tissues can be mutant, testing for the presence or absence of wild-type p53 protein can provide diagnostic and prognostic information regarding a tumor and the patient. The cells to be tested are typically isolated from a tissue suspected of being neoplastic. Preferably the tissues are carefully prepared and isolated so that non-neoplastic tissues are not mixed with the neoplastic tissues, which can confound the analysis. Means for separating neoplastic tissues from non-neoplastic tissues are known in the art and include dissection of paraffin or cryostat sections, as well as use of flow cytometry. A cell lysate can be prepared from the tumor tissue according to any method known in the art. The cell lysate is then incubated with DNA **fragments which are known to bind the wild-type p53** protein, under conditions which are conducive to such DNA/protein interactions. Alternatively, a histological sample can be analyzed by incubation with DNA fragments, as described for cell lysates.

Detail Description Paragraph - DETX (11):

[0074] According to another embodiment of the invention, after incubation of **p53 with specific binding DNA fragments** all components of the cell lysate which do not bind to the DNA fragments are removed. This can be accomplished, among other ways, by employing DNA fragments which are attached to an insoluble polymeric support such as agarose, cellulose and the like. After binding, all non-binding components can be washed away, leaving p53 bound to the DNA/solid

support. The p53 can be quantitated by any means known in the art. It can be determined using an immunological assay, such as an ELISA, RIA or Western blotting.

Detail Description Paragraph - DETX (12):

[0075] The diagnostic assay of the present invention has applicability not only with regard to cancers which are known to involve mutation of **p53, but also with regard to human** viruses such as human papilloma virus (HPV). HPV protein E6 binds tightly to wild-type but not mutant p53. See Werness et al., Science, 248, 76-69 (1990). This tight binding is likely to block the interaction of p53 with its specific DNA binding sequences. By testing cells or cell extracts suspected of being infected with potentially tumor-inducing or hyperplastia-inducing strains of HPV or possibly other viruses, infected cells can be identified, because the E6 protein of the infected cells will have sequestered the wild-type p53, rendering it unable to bind to its specific DNA binding sequences. Such assays may be performed on cell extracts or on histological specimens.

Detail Description Paragraph - DETX (13):

[0076] According to the present invention a method is also provided of supplying wild-type p53 function to a cell which carries mutant p53 alleles. The wild-type p53 gene or a part of the gene may be introduced into the cell in a vector such that the gene remains extra-chromosomal. In such a situation the gene will be expressed by the cell from the extrachromosomal location. If the mutant p53 genes present in the cell are expressed, then the wild-type **p53 gene or gene portion** should be expressed to a higher level than that of the mutant gene. This is because the mutant forms of the protein are thought to oligomerize with wild-type forms of the protein. (Eliyahu et al., Oncogene, vol. 3, p. 313, 1988.) If a gene **portion is introduced and expressed in a cell carrying a mutant p53 allele, the gene portion should encode a part of the p53** protein which is required for non-neoplastic growth of the cell. More preferred is the situation where the wild-type p53 gene or a part of it is introduced into the mutant cell in such a way that it recombines with the endogenous mutant p53 gene present in the cell. Such recombination would require a double recombination event which would result in the correction of the p53 gene mutation. Vectors for introduction of genes both for recombination and for extrachromosomal maintenance are known in the art and any suitable vector may be used.

Detail Description Paragraph - DETX (15):

[0078] According to the present invention compounds which have p53 activity are those which specifically complex with a p53-specific DNA binding site. Wild-type **p53 is one such compound, but portions of p53** which retain the ability to bind to p53-specific binding sites may also be used. Oligonucleotides and oligonucleotide containing nucleotide analogs are also contemplated among those compounds which are able to complex with a p53-specific DNA binding site. Although applicants do not wish to be bound by any particular theory, it is believed that oligonucleotides bind double-stranded DNA to form triplexes. Such triplexes have been shown to block transcription of certain genes, as well as protect the DNA binding sites from

the action of enzymes such as DNA methylases. Although originally such oligonucleotides were thought to require only or predominantly pyrimidines (cytosine and thymine), purines have also successfully been incorporated into triplex forming oligonucleotides. Particular oligonucleotides which may be used include: nucleotides 140-162 of SEQ ID NO:2, nucleotides 128-158 of SEQ ID NO: 1, nucleotides 114-123 of SEQ ID NO: 1, or portions thereof having at least ten nucleotides.

Detail Description Paragraph - DETX (19):

[0082] Double-stranded DNA **fragments which comprise a p53**-specific DNA binding site and are attached to an insoluble polymeric support are also contemplated by this invention. The support may be agarose, cellulose, polycarbonate, polystyrene and the like. Such supported fragments may be used in screens to identify compounds which bind to p53-specific DNA binding sites. Similarly, such supported fragments may be used to perform diagnostic tests on cell lysates from suspected tumor tissues. They may also be used in assays used to screen potential chemotherapeutic agents, as discussed infra.

Detail Description Paragraph - DETX (20):

[0083] Although any method can be employed which utilizes the p53-specific DNA binding sites of the present invention, two particular methods are disclosed for screening for additional compounds that bind to p53-specific DNA binding sites. According to one method a test compound is incubated with a supported DNA fragment, as described above. The amount of test compound which binds to the supported DNA fragment is determined. This determination can be performed according to any means which is convenient. For example, the amount of a compound which can be removed after incubation with the supported fragment can be compared to the amount originally applied. Alternatively, the test compound can be labelled and the amount which binds to the supported fragment can be assayed directly. In order to render this screening method more specific, soluble DNA **fragments which do not contain the p53** DNA binding sequence can be added to the incubation mixture. The soluble **fragments would not have the ability to specifically bind to p53** wild-type protein.

Detail Description Paragraph - DETX (21):

[0084] According to another screening method for compounds to simulate the specific DNA binding activity of **p53, test compounds are incubated with supported DNA fragments** as described above. However, in this method wild-type p53 protein is also added to the incubation mixture. The amount of **p53 protein which binds to the DNA fragment** is measured using methods as described above. The amount of p53 protein bound is compared to the amount which binds in the absence of the test compound. Any diminution of p53 binding which results from the presence of the test compound is presumptively due to the competition of the test compound with **p53 for the specific DNA binding sites of the supported fragments**. Direct binding of the test compound to the binding site fragments can be confirmed using the assay described above.

Detail Description Paragraph - DETX (24):

[0087] Compounds which have p53-specific DNA-binding activity, including wild-type **p53 pr tein, polypeptides c rresp nding t portions of wild-type p53** protein, oligonucleotides and oligonucleotide containing nucleotide analogues, as well as other organic molecules can also be administered to humans and animals as a pharmaceutical and therapeutic composition. Effective amounts will be administered to cause neoplastic cells to become less aggressively neoplastic or even to stop the growth of the neoplastic cells entirely. Generally, such amounts will be in the range of 10 ng to 10 .mu.g per dose per person or other animal. The therapeutic compounds can be prepared in any conventional pharmaceutical excipient, such as physiological saline or other physiologically compatible aqueous buffer. Typically, the compounds will be administered by injection, either intravenous or intramuscular. However, other administration methods as are known in the art and may be used to administer the compounds of the present invention.

Detail Description Paragraph - DETX (30):
[0093] In another embodiment of the invention, oligonucleotides can be isolated which restore to mutant p53 proteins the ability to bind to the consensus binding sequence or conforming sequences. Mutant p53 protein and random oligonucleotides are added to a solid support on which **p53-specific-binding DNA fragments** are immobilized. Oligonucleotides which bind to the solid support are recovered and analyzed. Those whose binding to the solid support is dependent on the presence of the mutant p53 protein are presumptively binding the support by binding to and restoring the conformation of the mutant protein.

Detail Description Paragraph - DETX (40):
[0101] Each clone was digested with an appropriate restriction endonuclease, end-labelled with .sup.32P, and incubated with p53 protein from a lysate of cells infected with a recombinant vaccinia virus expressing p53 protein. Labelled DNA **fragments which bound to p53** were then recovered by immunoprecipitation with monoclonal antibodies against p53. Of the more than 1400 restriction **fragments tested, only two bound reproducibly to p53** under the experimental conditions used: a 259 basepair Hinfl fragment (fragment A) of clone 772 C.sub.BE (Panel 2, FIG. 1A), and a 190 basepair Hinfl fragment (fragment B) of clone Lambda 5R (Panel 3, FIG. 1A); these fragments bound to a far greater extent than any of the other labelled fragments of larger or smaller size present in the same assay mixes.

Detail Description Paragraph - DETX (42):
[0102] This example demonstrates that the immunoprecipitation of **fragment A is dependent on both p53** protein and anti-p53 antibodies.

Detail Description Paragraph - DETX (44):
[0104] Lysates from cells infected with wild-type vaccinia virus (devoid of **p53) were not able to specifically immunoprecipitate fragment** A (FIG. 1B). Similarly, the detection of the precipitation of **fragment A was dependent n the presence f anti-p53** antibodies (FIG. 1B). The binding was evident in

lysates prepared from either human HeLa cells or monkey BSC40 cells infected with vaccinia virus and expressing wild-type p53 (FIG. 1B).

Detail Description Paragraph - DETX (45):

[0105] Affinity-purified baculovirus-produced wild-type p53 protein was substituted for the vaccinia-infected cell lysates in the immunoprecipitation assay and found to bind fragment A strongly (FIG. 2A). This suggested that the binding to **fragment A was an intrinsic property of the p53** polypeptide and not dependent on other factors present in the vaccinia virus-infected cell lysates.

Detail Description Paragraph - DETX (47):

[0106] The example demonstrates that **p53 mutant proteins found in human** tumors fail to bind to fragment A.

Detail Description Paragraph - DETX (48):

[0107] Increasing quantities of wild-type and mutant .sub.273his p53 protein, affinity purified from a baculovirus expression system, were used to immunoprecipitate labelled fragments from CBE. See FIG. 2A. The proportion of **fragment A bound to wild-type p53** protein increased in tandem with the amount of p53 added to the assay mixture. (FIG. 2A) In contrast, fragment A did not specifically bind to a mutant form of p53 (.sub.273his) protein even at the highest p53 protein concentration used. The 273.sup.his mutation is the most common **p53 mutant identified in human** tumors. Another p53 mutant (.sub.175his) protein commonly found in human tumors also failed to bind to fragment A (FIG. 1B).

Detail Description Paragraph - DETX (50):

[0108] This example defines the particular sequences within **fragment A that allow it to bind to wild-type p53** protein.

Detail Description Paragraph - DETX (52):

[0110] One primer for each PCR was labelled with .sup.32P at the 5' end with T4 polynucleotide **kinase** in a 5 .mu.l reaction, and the **kinase** inactivated at 70.degree. C. for 5 min. PCR contained 350 ng of each of the appropriate primers and approximately 50 ng plasmid template in a 50 .mu.l reaction, using 25 cycles and the PCR conditions specified in Baker S J, et al., Cancer. Res., 50:7717 (1990). The products were extracted with phenol and chloroform, ethanol-precipitated, and dissolved in 3 mM Tris, 0.2 mM EDTA prior to binding. Subfragment 1 contained bp 1 to 425 of subclone 10d of **fragment** A (FIG. 3A); subfragments 1a, 1b, 1c, 1d, and 1e were generated by digestion of subfragment 1 with BamHI, MboI, HindIII, HindIII, and BamHI, respectively, from **fragment** 1. Subfragment 2, contained bp 283 to 425. Subfragment 3a was generated by digestion of subfragment 3 (bp 106 to 294) with Hae III. Subfragment 4a was produced from subfragment 4 (gp 1 to 141) by Hind III digestion. Subfragments 5a and 5b were products of the HaeII digestion of subfragment 5 (bp 87 to 141). "Mutant" subfragments 5mut1 and 5 mut2 were produced using primers P3ml (5'-GAAAGAAAAGGCAAGGCCAGGAAAGT-3') and P3mut2

(5'-GAAAGAAAAGGCAAGGCCATTAAAGT-3') and were identical to subfragment 5 except for the positions underlined in the primers. Subfragment 6 contained bp 106 to 138, and the insert was excised by restriction with HindIII and BamHI to generate 6a or with HindIII and EcoRI to generate 6b. Subfragment 3, including basepairs 106 to 294 (FIG. 4B, panel 2) bound well to **p53** as did subfragment 4, containing basepairs 1 to 141 (FIG. 4B, panel 3). This and similar assays done with additional subfragments (FIGS. 4A and 4B) localized the critical sequences to basepairs 106 to 141. This segment contained three repeats of the sequence TGCCT (FIG. 3A). Digestion of subfragment 3 with HaeIII (cleaving between bp 125-126 and removing two of the TGCCT repeats) greatly reduced this binding (FIG. 4B, subfragment 3A, panel 2), suggesting that a critical sequence lay at or near this restriction site and that a single TGCCT repeat was not sufficient for binding. Additional subfragments were tested (#5, bp 87 to 141, FIGS. 4A and 5B; #6, bp 106 to 138, FIGS. 4A and 4B, panel 4), and it was established that a 33 bp insert containing three TGCCT repeats provided binding capability.

Detail Description Paragraph - DETX (54):

[0111] This example demonstrates that certain G residues are critical for binding of **p53 to fragment** A.

Detail Description Paragraph - DETX (58):

[0114] This example defines the region of **fragment B which is important for p53** binding.

Detail Description Paragraph - DETX (61):

[0116] This example shows that expression of the wild-type **p53 gene in human** colorectal carcinoma cells dramatically inhibits their growth and that a mutant **p53 gene cloned from a human** colorectal carcinoma was incapable of exerting such inhibition.

Detail Description Paragraph - DETX (69):

[0124] The conclusions made f rom the above experiments are dependent on the assumption that p53 protein was produced in the transfected cell lines. Clones containing exogenous mutant p53 sequences produced p53 mRNA at a concentration 1.5 to 3.5 times higher than that produced by the endogenous p53 gene (FIGS. 6A and 7A). Immunoblot analysis showed that there was a concomitant small increase in p53 protein expression in the transfectants (1.5- to 3-fold) compared to the untransfected cells. However, this increase was difficult to measure quantitatively, since these cells produced significant amounts of endogenous p53 protein that (unlike endogenous p53 mRNA) could not be distinguished from that produced by the vectors. To confirm that transfected **human cells expressed p53** protein from our constructs, we studied an additional colorectal carcinoma cell line (RKO). RKO cells were obtained through the generosity of M. Brattain. Although RKO cells did not contain a mutation within the susceptible p53 coding sequences, i.e., exons 5-9, they expressed low concentrations of p53 mRNA compared to normal colorectal mucosa or the other lines studied and did not produce detectable amounts of protein.

Detail Description Paragraph - DETX (70):

[0125] Results of colony formation assays in transfected RKO cells were similar to those in SW480 and SW837 cells. Colony formation by wild-type p53 gene transfectants occurred with a tenfold decrease in efficiency compared to the mutant p53 construct (Table 1). Immunocytochemical detection of p53 protein in transfected RKO cells was done as follows: approximately 5.times.10.sup.4 cells were cytocentrifuged onto polylysine-coated slides, fixed for 10 min in formalin, and permeabilized for 5 min in 0.5% Triton X-100. A mouse monoclonal antibody against **human p53** protein (Ab1801) in combination with the ABC immunoperoxidase system (Vector Laboratories), was used for immunocytochemical detection of p53 protein (Banks, et al., Eur. J. Biochem. 159, 529 (1986)). Ten to 20 randomly selected microscopic fields were analyzed per slide. These observations are consistent with the greater stability of mutant compared to wild-type p53 protein noted previously (C. A. Finlay et al., Mol. Cell Biol. 8, 531 (1988)). However, transient mRNA expression was also significantly lower in the SN3 transfectants compared to the SCX3 transfectants at 48 and 96 hours, supporting the idea that RKO cells expressing wild-type p53 were at a selective disadvantage compared to those producing mutant p53 products.

Detail Description Paragraph - DETX (76):

[0130] This example demonstrates the identification of human genomic **fragments that can bond to wt p53** protein in vitro.

Detail Description Paragraph - DETX (78):

[0132] Following the outline in FIG. 8A., we tested the inserts of 530 clones for binding to p53. Restriction fragments of the clones were end-labeled and incubated with purified **human wt p53** protein produced in baculovirus-infected cells.

Detail Description Paragraph - DETX (79):

[0133] Whole-genome PCR was performed as previously described, except that only one oligonucleotide (5'-GAGTAGAATTCTAATATCTC-3') was used for amplification (Kinzler, et al. (1989), Nucleic Acids Research, 17:3645-3653, and Kinzler, et al. (1990), Molec. Cell. Biol., 10:634-642). Two hundred ng of "catch"-linked human genomic DNA were incubated with 100 ng of baculovirus-produced **human wt p53** purified as described (Friedman, et al. (1990), Proc. Natl. Acad. Sci. U.S.A., 87:9275-9279), and immunoprecipitated as described below. After 4 rounds of IP and PCR, the AS DNA was cleaved with Eco RI and cloned into either the vector Lambda Zap II or pBluescript II SK+ (Stratagene). Individual clones were picked at random and tested for p53 binding. In panel B, cloned plasmid DNA samples were cleaved with Eco RI and end-labeled by Klenow fill-in. For IP (McKay, et al. (1981), J. Mol. Biol, 145:471-479), ten ng of DNA were incubated with 100 ng of baculovirus-produced **human wt p53** and 100 ng of poly dl-dC at 4.degree. C. for 30 minutes in 100 .mu.l of "DNA-binding buffer" containing 100 mM NaCl, 20 mM Tris, pH 7.0, 10% glycerol, 1% NP40, and 5 mM DTT. DNA **fragments bound to p53** were complexed to antibodies by the addition of 8 .mu.l containing 400 ng each of anti-p53 antibodies pAb421 and pAb1801, both obtained from Oncogene Science, and incubated for 30 minutes at 4.degree. C. The DNA-binding buffer containing

1.5 mg protein A precipitated following the addition of 26 .mu.l of DNA-binding buffer containing 1.5 mg protein A Sepharose and 10 .mu.g of poly dl-dC and mixing at 4.degree. C. for 30 minutes. After removal of the supernatant, the immunoprecipitate was washed twice with 1 ml of DNA-binding buffer. Bound DNA was purified by treatment with SDS and proteinase K at 48.degree. C. for 30 minutes, extracted with phenol and chloroform, precipitated with ethanol, separated by electrophoresis on a 10% nondenaturing polyacrylamide gel, and autoradiographed.

Detail Description Paragraph - DETX (80):

[0134] Twenty-three of the clones were found to contain **fragments that bound to p53**. Examples of the IP experiments are shown in FIG. 8B. Clone S61 (lanes 11B,C) contains a single genomic **fragment of 202 bp which bound to p53**. Clone N2 contained five **fragments, only one of which (357bp) bound to p53** (lanes 10B,C). Other examples of **p53-binding fragments** were obtained, and each of these was subcloned for further analysis. In contrast, we found that none of over 1000 clones containing unselected human DNA inserts of similar size bound to p53 using the IP assay. Thus, the whole-genome PCR procedure significantly enriched for p53-binding sequences.

Detail Description Paragraph - DETX (82):

[0135] This example demonstrates the localization of **p53 contacts with bound DNA fragments**.

Detail Description Paragraph - DETX (83):

[0136] Localization of the regions bound by p53 was obtained by DP or MI assays using the subcloned DNA fragments as probes. For MI, the **fragments were methylated at G residues and bound to p53** (FIG. 9). Methylation of G residues critical for p53 binding resulted in interference with IP. For example, methylation at nucleotides, 217, 22, 227 to 229, and 233 of the 248 bp insert from clone 11B3 completely interfered with the binding of this **fragment to p53** (FIG. 9, footprint 2). When the opposite strand was analyzed, interference was observed at the G residues corresponding to nucleotides 219, 223, 224, 230, 235, and 236 (FIG. 9, footprint 1). For DP, labelled DNA fragments were first subject to IP, then incubated with various amounts of DNase I. For clone N22, p53 binding provided protection against DNase I cleavage at residues 187 to 211 (FIG. 9, footprint 9). MI showed interference by G residues only within the region protected by DNase I (FIG. 9, footprint 10). Other examples of DP and MI mapping are shown in FIG. 9. **p53-binding DNA fragments** were subcloned and labeled on one end, gel-purified and subjected to DP or MI mapping. For MI, 10 ng of DNA were incubated in 200 .mu.l of 50 mM Na-cacodylate, 1 mM EDTA, pH 8.0 and 5 .mu.l of 10% dimethylsulfate/90% ethanol for 5 minutes at 20.degree. C. to methylate G residues. Fifty .mu.l containing 1.5 m Na-acetate, 1 M .beta.-mercaptoethanol and 60 .mu.g of glycogen were added. The mixture was ethanol-precipitated, washed, and resuspended in 5 .mu.l of 3mM Tris, 0.2 mM EDTA, pH 7.5, and allowed to bind to wild-type p53 as described in the legend to FIG. 1. After IP and DNA purification, the samples were incubated with 100 .mu.l of I M piperidine at 90.degree. C. for 30 minutes. The samples were then dried under vacuum and separated electrophoretically on a 6%

polyacrylamide sequencing gel. The control DNA samples were carried through all incubations except no p53 was added. For these control samples, the protein A Sepharose pellets were treated with SDS and proteinase K without removal of the supernatants (which contained the labeled DNA in the absence of p53).

Detail Description Paragraph - DETX (84):

[0137] For DP assays, end-labeled DNA fragments were immunoprecipitated as described in the legend to FIG. 8. The protein A Sepharose pellets were incubated for two minutes at 25.degree. C. with 200 ng DNase I in 5 mM MgCl.sub.2. After purification of the DNA, as described above, samples were separated by electrophoresis on sequencing gels and loaded as described above for MI. MI was performed on all 18 genomic DNA **fragments which bound to p53**. DP assays were performed on 13 fragments and the regions of protection uniformly coincided with those indicated by the MI assays.

Detail Description Paragraph - DETX (94):

[0144] This example demonstrates that intact **p53 can activate expression in human** cells.

Detail Description Paragraph - DETX (95):

[0145] We first made reporter plasmids (PG.sub.n-CAT series) containing part of the polyomavirus early promoter and the CAT gene located downstream of DNA sequences which could bind to **p53** in vitro (FIG. 8). For the CAT reporters, concatemers of the **p53**-binding region of C.sub.BE were formed by ligation of complementary oligonucleotides, ligated into the EcoRV site of pBluescript II SK+ (Stratagene) to form the PG.sub.n and MG.sub.n series. The BglII-BamHI **fragment** of pPyOICAT (Murakami, et al. (1990) Oncogene, 5:5), containing the polyomavirus early promoter and the CAT gene coding region, was ligated into the BamHI site of the PG.sub.n and MG.sub.n series clones to form the PG.sub.n-CAT and MG.sub.n-CAT series, and the orientation of the inserts characterized by restriction enzyme analysis. The PG.sub.9-MG.sub.n-CAT and PG.sub.13-MG.sub.n-CAT series were formed by excising the HindIII-SalI **fragments** of PG.sub.9-CAT and PG.sub.13-CAT, blunt-ending, attaching XbaI linkers, and ligating into the XbaI site of the MG.sub.n-CAT series plasmids (where n=1, 5, 10, and 15). For the yeast .beta.-galactosidase reporter plasmids, PG and MG sequences were ligated as SalI-SmaI **fragments** to the SalI and filled-in XhoI sites of pCZ (Buchanan, et al. (1988), Mol. Cell Biol., 8:50806). The construction of the **p53**-wt expression construct has been described (Baker, et al. (1990), Science, 249:912); the mutant expression plasmids were constructed similarly from the previously described cDNA plasmids (Nigro, et al. (1989), Nature, 342:705, and Kern, et al. (1991), Oncogene, 6:131), or in the case of the engineered **phosphorylation** site mutants, by in vitro mutagenesis (Altered Sites, Promega) with verification by sequencing. The construction of the yeast **p53** expression vectors based on pRS314 has been described (Nigro, et al., Mol. Cell Biol. (in press)).

Detail Description Paragraph - DETX (96):

[0146] For the p53 binding sequences, we used a series of concatemers of the oligonucleotide PG (5'-CCTGCCTGGACTTGCCTGG-3'). This contained. the binding region of plasmid C.sub.BE. previously shown to bind p53 in vitro. The reporter and an expression vector coding for the intact **human wild-type protein (p53**-wt) (FIG. 12B), were transfected together into the human colorectal cancer cell line HCT 116. This line makes low amounts of apparently wild-type p53 protein.

Claims Text - CLTX (1):
1. A method for detecting the presence of wild-type p53 protein in a cell, comprising the steps of: contacting a **p53-specific binding DNA fragment** with a cell lysate from a tissue of a human, to bind the DNA **fragment to wild-type p53** present in the cell lysate; detecting the presence of wild-type **p53 protein in the cell by detecting binding of the DNA fragment to wild-type p53**.

Claims Text - CLTX (4):
4. The method of claim 1 wherein the **p53-specific binding DNA fragment** comprises more than one monomer of the sequence 5'-rrrcwwgyyy-3'.

Claims Text - CLTX (8):
8. A method of detecting the presence of a wild-type p53 protein in a cell, comprising the steps of: providing a histological section from a human; incubating the section with a detectably-labeled **p53-specific binding DNA fragment to bind said DNA fragment to wild-type p53** present in the histological sample; removing unbound DNA fragment from the histological section; and determining the amount of DNA fragment which is bound to the histological sample.

Claims Text - CLTX (11):
11. The method of claim 8 wherein the **p53-specific binding DNA fragment** comprises more than one monomer of the sequence 5'-RRRCWWGYYY-3'.

Claims Text - CLTX (17):
17. The method of claim 16 wherein the polypeptide comprises all or a part of **human wild-type p53** protein.

Claims Text - CLTX (22):
22. A double-stranded DNA **fragment which comprises a p53-specific DNA binding site, wherein the fragment** comprises more than one monomer of the sequence RRRCWWGYYY and wherein the fragment is covalently attached to an insoluble polymeric support.

Claims Text - CLTX (28):
28. A method of identifying compounds which specifically bind to p53-specific DNA binding sequences, comprising the steps of: contacting a

p53-specific-binding DNA fragment with a test compound to bind the test compound to the DNA fragment; determining the amount of test compound which is bound to the DNA fragment.

Claims Text - CLTX (29):

29. The method of claim 28 wherein soluble DNA **fragments are incubated with the test compound and the p53-specific-binding DNA fragment** immobilized on a solid support, said soluble DNA **fragments not having the ability to specifically bind wild-type p53** protein.

Claims Text - CLTX (30):

30. A method of identifying compounds which specifically bind to p53-specific DNA binding sequences, comprising the steps of: contacting a **p53-specific-binding DNA fragment** immobilized on a solid support with both a test compound and wild-type p53 protein to bind the wild-type **p53 protein to the DNA fragment**; determining the amount of wild-type **p53 protein which is bound to the DNA fragment, inhibition of binding of wild-type p53** protein by the test compound indicating binding of the test compound to the p53-specific DNA binding sequences.

Claims Text - CLTX (34):

34. A method of supplying a wild-type p53 gene function to a cell which has lost said gene function by virtue of a mutation in a **p53 gene, comprising: introducing a portion of a wild-type p53 gene into a cell which has lost said gene function such that said portion is expressed in the cell, said portion encoding a part of the p53** protein which is required for non-neoplastic growth of said cell.

Claims Text - CLTX (39):

39. A method of diagnosing tumor-inducing or hyperplastic-inducing strains of human papilloma virus (HPV) comprising: contacting cells or cell extracts of patients suspected of being infected by HPV with a **p53-specific binding DNA fragment**; detecting the amount of wild-type **p53 in said cells or cell extract which binds to said DNA fragment, absence of bound p53** indicating infection by strains of HPV which sequester p53.

Claims Text - CLTX (41):

41. A method of pre-screening oligonucleotides for use in cancer therapy, comprising: adding a p53 protein which is encoded by a mutant gene found in a cancer patient and a preparation of random oligonucleotides to a **p53-specific-binding DNA fragment** immobilized on a solid support; recovering the olignucleotides which bound to the solid support.

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ABSTRACT:

The invention provides a method of diagnosing cancer by determining the expression level or gene amplification of p53 and dm2, whereby an elevated level of either p53 or dm2 or both p53 and dm2 indicates a cancer diagnosis. Furthermore, the invention provides a method of predicting the progress of cancer by determining the expression level or gene amplification of p53 and dm2, whereby an elevated level of either p53 or dm2 or both 53 and dm2 indicates a poor prognosis.

[0001] This application is a continuation-in-part of PCT Application No. _____, filed Jun. 25, 1993, which is a continuation-in-part of U.S. Ser. No. 08/018,649, filed Feb. 17, 1993, which is a continuation-in-part of U.S. Ser. No. 07/904,766, filed Jun. 26, 1992, which in turn is a continuation-in-part of U.S. Ser. No. 07/730,185, filed Jul. 12, 1991, which in turn is a continuation-in-part of U.S. Ser. No. 07/543,963 filed Jun. 27, 1990, all of which are incorporated herein by reference.

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Summary of Invention Paragraph - BSTX (12):

[0012] The human homolog of the mdm2 gene, called the hdm2 gene or MDM2 or MDM2, has been cloned and mapped to the long arm of chromosome 12 (12q13-14) (Oliner et al. 1992. Amplification of a gene encoding a **p53-associated protein in human** sarcomas. Nature 358:80-83). This region contains two genes, SAS and GLI, previously found to be amplified in osteo- and soft tissue sarcomas. The SAS gene codes for a protein of unidentified function. It was isolated from a malignant fibrous histiocytoma (MFH), and was shown to be amplified in MFH and liposarcomas (Turc-Carel, C. et al. (1986) Cancer Genet Cytogenet 23, 291-299; Meltzer, P. S. et al. (1991) Cell Growth Diff 2, 495-501). The GLI gene codes for a DNA-binding zinc finger protein. Even though it was originally isolated from a glioblastoma, it has also been reported to be amplified in a rhabdomyosarcoma and an osteosarcoma (Kinzler, K. et al.(1984) Science 236, 70-73).

Detail Description Paragraph - DETX (35):

[0056] Briefly, polyclonal antibodies may be produced by injecting a host mammal, such as a rabbit, mouse, rat, or goat, with the **p53 protein or a fragment** thereof capable of producing antibodies that distinguish between mutant p53 and wild-type p53. The peptide or peptide fragment injected may contain the wild-type sequence or the mutant sequence. Sera from the mammal are extracted and screened to obtain polyclonal antibodies that are specific to the peptide or peptide fragment. The same method may be applied to dm2 proteins.

Detail Description Paragraph - DETX (39):

[0060] Suitable antibodies for the co-immunoprecipitation of p53 and dm2 include PAb421 and Ab2. PAb421 recognizes the carboxy-terminus of **p53 fr m various species, including human, mouse and rat p53,** and is described by Harlow

et al. in the Journal of Virology 39, 861-869 (1981). Ab2 is specific for the amino-terminus of **human p53,** and is available from Oncogene Science, Inc. of Manhassett, N.Y. The dm2 protein does not immunoprecipitate when REF cells that do not express p53 are treated in the same way with the same antibodies.

Detail Description Paragraph - DETX (69):

[0088] A .lambda.gt11 library constructed from HeLa cells was screened using the mouse mdm2 cDNA under conditions of reduced stringency. A total of 14 positive clones were isolated and the CDNA inserts subcloned into the Bluescript vector for further analysis. Preliminary restriction mapping and partial sequencing showed that they represent partial clones for the human dm2 cDNA (Fakharzadeh, S. S. et al. 1991. Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. EMBO J. 10:1565-1569). A full length coding region was constructed from two overlapping cDNA clones and sequenced. The DNA sequence of this CDNA clone, designated hdm2, is similar to the published hdm2 sequence (Oliner, J. D. et al. 1992. Amplification of a gene encoding a **p53-associated protein in human** sarcomas. Nature 358:80-83), with complete identitity within the coding region and a few differences in the noncoding regions. The fact that these two CDNA clones were obtained from two very different sources (HeLa cell vs. colon carcinoma), yet have identical coding sequences, suggests that they may represent the wild-type hdm2 coding sequence or a systematic mutation is present in different cancer cells.

Detail Description Paragraph - DETX (76):

[0094] A panel of mouse monoclonal antibodies to the p90 gene encoded gene product were used for the present study. Antibody 4B2 detects an epitope located in the amino**-terminal** region. Antibodies 2A9 and 2A10 identify two distinct epitopes in the central **portion** of p90. Antibody 4B11 recognizes a sequence located in the carboxy**-terminal** region of p90. Three mouse monoclonal antibodies detecting different epitopes on **p53** proteins were used for the present study. Anti**-p53** antibody PAbl80l (Ab-2, Oncogene Science, Manhasset, N.Y.) recognizes an epitope located between amino acids (aa) 32 to 79 of both wild-type and mutant **human p53** proteins (Banks, L. et al. (1986) Eur J Biochem 159, 529-534). Antibody PAb240 (Ab-3, Oncogene Science) recognizes a conformational epitope located between aa 156 to 335 characteristic of certain mutant **p53** products (Gannon, J. V. et al. (1990) EMBO J 9, 1595-1602). Antibody PAbl620 (Ab-5, Oncogene Science) reacts specifically with wild type **p53** (Ball, R. K. et al. (1984) EMBO J 3, 1485-1491). MIgS-Kp I, a mouse monoclonal antibody of the same subclass as the anti-p90 and anti**-p53** antibodies, was used as a negative control at similar working dilutions.

Detail Description Paragraph - DETX (82):

[0100] These studies were performed according to a slight modification of the method reported by Orita et al (Orita, M. et al. (1989) Genomics 5, 874-879). Amplifications were performed using 100 ng of genomic DNA extracted from the samples described above. The primers used were obtained from intronic sequences flanking exons 5 through 9 of the **human p53** gene, sequences being previously published (Moll, U. M. et al. (1992) Proc Natl Acad Sci USA 89,

7262-7266). DNA was amplified following 30 cycles of PCR (30s at 94.degree. C., 30s at 58.degree. C. for exons 8 and 9 and 63.degree. C. for exons 5, 6 and 7, and finally 60s for all samples at 72.degree. C.) using a Thermal Cycler (Perkin Elmer Cetus). Amplified samples were then denatured and loaded onto a non-denaturing acrylamide gel containing 10% glycerol and run at room temperature for 12-16 hours at 10-12 watts. Gels were dried at 80.degree. C. under vacuum and exposed to X-ray film at -70.degree. C. for 4-16 hours.

US-PAT-NO: 6537594

DOCUMENT-IDENTIFIER: US 6537594 B1

TITLE: Vaccina virus comprising cytokine and/or tumor
                 associated antigen genes

DATE-ISSUED: March 25, 2003

INVENTOR-INFORMATION:
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APPL-NO: 09/ 535370

DATE FILED: March 24, 2000

PARENT-CASE:

CROSS REFERENCE TO RELATED APPLICATIONS

   This application is a continuation of application Ser. No. 08/460,736,
filed Jun. 2, 1995 now U.S. Pat. No. 6,265,189; which is a division of
application Ser. No. 08/184,009, filed Jan. 19, 1994 U.S. Pat. No.
5,833,975. Application Ser. No. 08/184,009 is a continuation-in-part of
application Ser. No. 08/007,115, filed Jan. 21, 1993, abandoned, incorporated
herein by reference. Application Ser. No. 08/007,115 is a
continuation-in-part of application Ser. No. 07/847,951, filed Mar. 6, 1992,
abandoned, which in turn is a continuation-in-part of application Ser. No.
07/713,967, abandoned, filed Jun. 11, 1991 which in turn is a
continuation-in-part of application Ser. No. 07/666,056, filed Mar. 7, 1991,
abandoned; and, application Ser. No. 08/007,115 is also a continuation-in-part
of application Ser. No. 07/805,567, filed Dec. 16, 1991, U.S. Pat. No.
5,378,457, which in turn is a continuation-in-part of application Ser. No.
07/638,080, filed Jan. 7, 1991, abandoned; and, application Ser. No.
08/007,115 is also a continuation-in-part of application Ser. No. 07/847,977
filed Mar. 3, 1992, abandoned, as a division of application Ser. No.
07/478,179, filed Feb. 14, 1990, abandoned, as a continuation-in-part of
application Ser. No. 07/320,471, filed Mar. 8, 1989, U.S. Pat. No.
5,155,020; all of which are hereby incorporated herein by reference. Reference
is also made to copending U.S. applications Ser. No. 715,921, filed Jun. 14,
1991, abandoned, U.S. application Ser. No. 736,254, filed Jul. 26, 1991,
abandoned, U.S. application Ser. No. 776,867, filed Oct. 22, 1991,
abandoned, and U.S. application Ser. No. 820,077, filed Jan. 13, 1992,
abandoned, all of which are hereby incorporated herein by reference.

US-CL-CURRENT: 424/93.2, 424/191.1 , 424/93.6 , 435/320.1 , 435/69.1
       , 435/69.3 , 435/69.5 , 435/69.51 , 435/69.52 , 435/70.1

, 435/70.3 , 514/44

ABSTRACT:

Attenuated recombinant viruses containing DNA coding for a cytokine and/or a tumor associated antigen, as well as methods and compositions employing the viruses, are disclosed and claimed. The recombinant viruses can be NYVAC or ALVAC recombinant viruses. The DNA can code for at least on of: human tumor necrosis factor; nuclear phosphoprotein **p53, wildtype or mutant; human** melanoma-associated antigen; IL-2; IFN.gamma.; IL-4; GNCSF; IL-12; B7; erb-B-2 and carcinoembryonic antigen. The recombinant viruses and gene products therefrom are useful for cancer therapy.

23 Claims, 46 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 33

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Abstract Text - ABTX (1):

Drawing Description Text - DRTX (41):
FIG. 39 shows the coding sequence for the **human p53** gene (SEQ ID NO:215).

Detailed Description Text - DETX (287):
Generation of Poxvirus-based Recombinant Viruses Expressing Wildtype and Mutant Forms of the **Human p53** Gene Product

Detailed Description Text - DETX (288):
Three plasmids, p53wtXbalSP6/T3, p53-217Xbal, and **p53-238Xbal containing wildtype human p53** gene sequences, and two mutant forms of p53, respectively, were obtained from Dr. Jeffrey Marks (Duke University). The p53-217Xbal contains a p53 gene encoding a p53 product lacking codon 217 while p53-238Xbal encodes a p53 gene product with an cysteine to arginine substitution at amino acid 238. The sequence of the wildtype p53 cDNA and the deduced amino acid sequence was described previously (Lamb and Crawford, 1986; FIG. 3).

Detailed Description Text - DETX (290):
    PCR was also used to generate a 480 bp and 250 bp fragment from
p53wtXbalSP6/T3. The 480 bp fragment was derived with oligonucleotides MM003
(SEQ ID NO:92) (5'-GTTTGTATCGTAATGGAGGAGCCGCAGTCAGATC-3') and MM008 (SEQ ID
NO:93)
(5'-CATTACGATACAAACTTAACGGATATCGCGACGCGTTCACACAGGGCAGGTCTTGGC-3')
This
fragment contains the 3' portion of the vaccinia virus H6 promoter sequences
and the 5' **portion of the p53** coding sequences through the SgrAl site. The 250
bp fragment was derived by amplification with oligonucleotides MM005 (SEQ ID
NO:94) (5'-TACTACCTCGAGCCCGGGATAAAAAACGCGTTCAGTCTGAGTCAGGCCC-3') and
MM007.
(SEQ ID NO:95)
(5'-GTGTGAACGCGTCGCGATATCCGTTAAGTTTGTATCGTAATGCAGCTGCGTGGGCGTGA
.
This PCR **fragment contains the 3' end of the p53** coding sequences beginning at
the Stul restriction site. The 480 bp and 250 bp PCR fragments were generated
such that the 5' end of the MM005/MM007-derived (SEQ ID NO:94/95) fragment
overlaps the 3' end of the MM003/MM008-derived (SEQ ID NO:92/93) fragment.

Detailed Description Text - DETX (291):
    The 227 bp, 480 bp, and 250 bp PCR-derived **fragments** were pooled and fused
by PCR using oligonucleotides MM006 (SEQ ID NO:96)
(5'-ATCATCGGATCCCCCGGGTTCTTTATTCTATAC-3') and MM005 (SEQ ID NO:94). The
783 bp
fused PCR product contains the H6 promoter juxtaposed 5' to the 5' **portion of
the p53** coding sequence (through the SgrAl restriction site) followed by the
end of the **p53** coding sequence beginning at the Stul site. Following the end
of the **p53** coding sequence, a T.sub.5 NT sequence motif providing early
vaccinia transcription **termination** (Yuen and Moss, 1986) and a unique XhoI site
were added. It should be noted that the final H6-**p53** PCR fusion product (783
bp) does not contain the **p53** coding sequences between the SgrAl and Stul
restriction sites.

Detailed Description Text - DETX (293):
    Plasmids containing intact p53 gene (wildtype or mutant forms) juxtaposed 3'
to the H6 promoter were generated by first digesting pMM105 with SgrAl and
Stul. A 795 bp SgrAl/Stul **fragment was isolated from p53wtXbalSP6/T3 and
p53-238Xbal, while a 792 bp fragment was isolated from p53**-217Xbal. These
fragments were individually ligated to the SgrAl/Stul digested pMM105 plasmid
to yield pMM106, pMM108, and pMM107, respectively.

Detailed Description Text - DETX (296):
    ALVAC (CPpp) p53 insertion plasmids were engineered by excising the p53
expression cassettes from pMM106, pMM107, and pMM108 by digestion with BamHI
and XhoI and inserting them individually into BamHI/XhoI digested pNVQC5LSP-7.
The 1320 bp BamHI/XhoI **fragment containing the H6-p53** expression cassette from
pMM106 and pMM108 was inserted into pNVQC5LSP-7 to yield pMM110 and pMM112,

respectively, while the 1317 bp BamHI/XhoI fragment derived from pMM107 and inserted into pNVQC5LSP-7 yielded pMM111.

Detailed Description Text - DETX (496):
   A **fragment containing the H6 promoted 5' end of the p53** gene fused to the 3' end of the p53 gene was generated by several PCRs as described below.

Detailed Description Text - DETX (498):
   PCR II: Plasmid p11-4 was used as template with oligonucleotides MM082 (SEQ ID NO:210) 5' CGTTAAGTTTGTATCGTAATGACTGCCATGGAGGAGTC 3' and MM083 (SEQ ID
NO:211) 5' TAGTAGTAGTAGTAGCTTCTGGAGGAAGTAGTTTCC 3' to generate a 129bp fragment
with the 3'-end of the H6 promoter, the 5' end of the **p53 gene followed by 15bp which overlaps PCR fragment** PCRIII (described below). MM082 contains the 3' end of the H6 promoter and primes from the 5' end of the murine p53 gene. MM083 anneals to position 97 (FIG. 38) of the murine p53 gene and primes toward the 5' end.

Detailed Description Text - DETX (499):
   PCRIII: Plasmid p11-4 was used as template with oligonucleotides MM084 (SEQ ID NO:212) 5' CAGAAGCTACTACTACTACTACCCACCTGCACAAGCGCC 3' and MM085 (SEQ ID
NO:213) 5' AACTACTGTCCCGGGATAAAAATCAGTCTGAGTCAGGCCCCAC 3' to generate a 301bp
fragment. The 301 bp PCR-derived **fragment contains the 3' end of the p53** gene, and the 5' end overlaps the 3' end of the PCRII product. MM084 (SEQ ID NO:212) primes from position 916 of the murine p53 gene toward the 3' end. MM085 (SEQ ID NO:) primes from position 1173 toward the p53 gene 5' end. The three PCR products were pooled and primed with MM080 and MM085. The resultant 588bp fragment contains a BamHI site followed by the H6 promoted 5' end of the p53 gene fused to the p53 gene 3' end followed by a SmaI site; the 5' end of the p53 gene ends at the XhoI site at position 37, and the 3' end starts at the SacII site at position 990 (FIG. 38). The 588bp PCR-derived fragment was digested with BamHI and SmaI generating a 565bp fragment which was inserted into BamHI/SmaI digested pNC5LSP5 (described below). The resultant plasmid, designated pMM136, was digested with KspI and XhoI to remove a 149bp fragment, and the 953bp KspI/XhoI fragment from p11-4 was inserted. The resultant plasmid, pMM148, contains the H6 promoted wild-type murine p53 in the ALVAC C5 insertion locus.

Detailed Description Text - DETX (507):
   Insertion of Mutant Forms of **Human P53** Into ALVAC and NYVAC

Detailed Description Text - DETX (508):
   Mutant forms of **Human p53** into ALVAC. FIG. 18 (Example 15) presented the sequence of the vaccinia H6 promoted **human wild type p53** gene cassette in an ALVAC-based recombinant, vCP207. In this example, to facilitate description of

the mutant forms of the **human p53** gene being described, FIG. 39 (SEQ ID NO:215) presents only the coding sequence for the **human wild type p53** gene. The start codon is at position 1 and the stop codon is at position 1180.

Detailed Description Text - DETX (509):
   Plasmid Cx22A, containing a mutant form of the **human p53** gene, was received from Arnold Levine (Princeton University, Princeton, N.J.). Relative to the wild type p53 sequence presented in FIG. 39, the G at nucleotide position 524 is substituted with an A, changing the arg amino acid at codon 175 of the wild type protein to a his amino acid in Cx22A.

Detailed Description Text - DETX (510):
   Plasmid pMM110 (Example 15, FIG. 18) contains the vaccinia H6 promoted wildtype **human p53** gene in the ALVAC C5 insertion site. The **human p53** gene contains two Pflml sites. p53 coding sequences upstream from the first Pflml site and downstream from the second Pflml site are the same in pMM110 as in Cx22A. pMM110 was digested with Pflml to remove the 853 central base pairs of the p53 gene. The 853bp Pflml fragment from Cx22A containing the base change at position 524 was inserted. The resultant plasmid, pMM143, contains the H6 promoted mutant p53 gene.

Detailed Description Text - DETX (511):
   Recombination between donor plasmid pMM143 and ALVAC rescuing virus generated recombinant virus vCP270. vCP270 contains the mutant form of the **human p53** gene under the control of the vaccinia H6 promoter in the C5 locus.

Detailed Description Text - DETX (512):
   Plasmid pR4-2 containing a mutant form of the **human p53** gene was received from Arnold Levine (Princeton University, Princeton, N.J.). Relative to the wild type p53 sequence presented in FIG. 39, the G at nucleotide position 818 is substituted by an A, changing the arg codon at amino acid position 273 to a his codon in pR4-2.

Detailed Description Text - DETX (513):
   Plasmid PMM110 (Example 15, FIG. 18) contains the vaccinia H6 promoted **human wildtype p53** gene in the ALVAC C5 insertion site. p53 coding sequences upstream from the first Pflml site and p53 coding sequences downstream from the second Pflml site are the same in pMM110 as in pR4-2. pMM110 was digested with Pflml to remove the 853 central base pairs of the p53 gene. The 853bp Pflml fragment from pR4-2 containing the base change at nucleotide position 818 was inserted. The resultant plasmid, pMM144, contains the H6 promoted mutant form of the **human p53** gene in the C5 insertion locus.

Detailed Description Text - DETX (514):
   Recombination between donor plasmid pMM144 and ALVAC rescuing virus generated recombinant virus vCP269. vCP269 contains the mutant form of the

human p53 gene under the control of the vaccinia H6 promoter in the C5 locus.

Detailed Description Text - DETX (515):
   Mutant forms of **Human P53** into NYVAC. Plasmid Cx22A, described above, contains a mutant form of the **human p53** gene, in which the G at nucleotide position 524 (FIG. 39) is substituted by an A, changing the arg codon at amino acid position 175 to a his codon in Cx22A.

Detailed Description Text - DETX (516):
   Plasmid pMM106 (Example 15) contains the vaccinia H6 promoted wild-type **human p53** gene in the NYVAC I4L insertion locus. p53 coding sequences upstream from the first PflmI site and p53 coding sequences downstream from the second PflmI site are the same in pMM106 as in Cx22A. pMM106 was digested with PflmI to remove the 853 central base pairs of the p53 gene. The 853bp PflmI fragment from Cx22A containing the base change at position 524 was inserted. The resultant plasmid, pMM140, contains the H6 promoted mutant p53 gene.

Detailed Description Text - DETX (517):
   Recombination between donor plasmid pMM140 and NYVAC rescuing virus generated recombinant virus vP1234. vP1234 contains the mutant form of the **human p53** gene under the control of the vaccinia H6 promoter in the I4L locus.

Detailed Description Text - DETX (518):
   Plasmid pR4-2, described above, contains a mutant form of the **human p53** gene, in which the G at nucleotide position 818 (FIG. 39) is substituted by an A, changing the arg codon at amino acid position 273 to a his codon in pR4-2.

Detailed Description Text - DETX (519):
   pMM106 (Example 15) contains the H6 promoted wild-type **human p53** gene in the I4L locus. p53 coding sequences upstream from the first PflmI site and p53 coding sequences downstream from the second PflmI site are the same in pMM106 as in pR4-2. pMM106 was digested with PflmI to remove the 853 central base pairs of the p53 gene. The 853bp PflmI fragment from pR4-2 containing the base change at position 818 was inserted. The resultant plasmid, pMM141, contains the H6 promoted mutant p53 gene.

Detailed Description Text - DETX (520):
   Recombination between donor plasmid pMM141 and NYVAC rescuing virus generated recombinant virus vP1233. vP1233 contains the mutant form of the **human p53** gene under the control of the vaccinia H6 promoter in the I4L locus.

Detailed Description Text - DETX (521):
   A listing of the wildtype and mutant forms of murine **p53 and the mutant forms of human p53** present in ALVAC and NYVAC recombinants described in Examples 31 and 32 is provided in Table 27.

Detailed Description Text - DETX (522):

Immunoprecipitation. ALVAC and NYVAC based recombinants vP1101, vP1096, vP1098, vCP207, VCP193, vCP191 (all described in Example 15; Table 22, as well as ALVAC and NYVAC based recombinants vCP270, vCP269, vP1233, vP1234 described in this Example, Table 27), contain wild type or mutant forms of the **human p53** gene. All of these recombinant virus were assayed for expression of the **human p53** gene using immunoprecipitation.

Detailed Description Text - DETX (523):

Recombinant or parental virus were inoculated onto preformed monolayers of tissue culture cells in the presence of radiolabelled .sup.35 S-methionine and treated as previously described (Taylor et al., 1990). Immunoprecipitation reactions were performed using a **human p53** specific monoclonal antibody 1801. A protein of between 47 and 53 kDa was precipitated from cells infected with any of the recombinant viruses, vP1101, vP1096, vP1098, vCP207, vCP193, vCP191, vCP270, vCP269, vP1233, or vP1234, but not from uninfected cells or cells infected with parental ALVAC or NYVAC virus.

Detailed Description Paragraph Table - DETL (37):

TABLE 27 Recombinant Parent Virus Virus Species Gene Insert vCP263 ALVAC murine w.t. p53 vCP267 ALVAC murine p53 (+3 aa) vCP270 ALVAC **human p53** (aa 175; R to H) vCP269 ALVAC **human p53** (aa 273; R to H) vP1234 NYVAC **human p53** (aa 175; R to H) vP1233 NYVAC **human p53** (aa 273; R to H)

US-PAT-NO: 6399755

DOCUMENT-IDENTIFIER: US 6399755 B1
See image for Certificate of Correction

TITLE: Products for inhibiting expression of human MDM2

DATE-ISSUED: June 4, 2002

INVENTOR-INFORMATION:
NAME	CITY	STATE	ZIP CODE	COUNTRY
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Vogelstein; Bert	Baltimore	MD	N/A	N/A

APPL-NO: 09/ 170159

DATE FILED: October 13, 1998

PARENT-CASE:

   This application is a continuation application U.S. Ser. No. 08/390,474,
filed Feb. 17, 1995, which is a divisional of U.S. Ser. No. 08/044,619,
filed Apr. 7, 1993, now U.S. Pat. No. 5,420,263 which is a
continuation-in-part of U.S. Ser. No. 07/903,103, filed Jun. 23, 1992, now
U.S. Pat. No. 5,411,860 which is a continuation-in-part of U.S. Ser. No.
07/867,840, filed Apr. 7, 1992, now abandoned.

US-CL-CURRENT: 536/23.1, 435/6

ABSTRACT:

   A human gene has been discovered which is genetically altered in human tumor
cells. The genetic alteration is gene amplification and leads to a
corresponding increase in gene products. Detecting that the gene, designated
hMDM2, has become amplified or detecting increased expression of gene products
is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and
allows the cell to escape from p53-regulated growth.

11 Claims, 22 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 17

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Abstract Text - ABTX (1):
   A human gene has been discovered which is genetically altered in human tumor

cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and allows the cell to escape from p53-regulated growth.

Brief Summary Text - BSTX (6):
   While there exists an enormous body of evidence linking **p53 gene mutations to human** tumorigenesis (Hollstein et al., 1991, Science 253:49-53) little is known about cellular regulators and mediators of p53 function.

Brief Summary Text - BSTX (7):
   Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated ras oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253:49-53. The transformed fibroblasts were found to produce elevated levels of **human p53** protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Brief Summary Text - BSTX (8):
   Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type **human p53** gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

Brief Summary Text - BSTX (9):
   Hinds et al., supra also expressed **human p53** protein in transformed rat cells. When the expressed **human p53** was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified M, 90,000 protein was coimmunoprecipitated. This suggested that the rat M, 90,000 protein is in a complex with the **human p53** protein in the transformed rat cell line.

Brief Summary Text - BSTX (19):
   Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (22):
   Still another object of the invention is to provide polypeptides which

interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (23):
   It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to **human p53** in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

Drawing Description Text - DRTX (7):
   FIG. 6 shows the determination of MDM2 and p53 domains of interaction. FIG. 6A and FIG. 6B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive .beta.-galactosidase reporter). Yeast clones expressing .beta.-galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined. .beta.-galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. FIG. 6C. Random **fragments of p53** were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Detailed Description Text - DETX (13):
   It has been found that amino acid residues 1341 of **p53** (See SEQ ID NO: 1) are necessary for the interaction of MDM-2 and **p53**. However, additional residues on either the amino or carboxy **terminal** side of the **peptide** appear also to be required. Nine to 13 additional **p53** residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from **p53**-regulated growth control in sarcomas, the use of **p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53**-regulated growth control.

Detailed Description Text - DETX (14):
   Suitable **p53-derived peptides** for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

Detailed Description Text - DETX (15):
   DNA encoding the MDM2-binding, **p53-derived peptide,** or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a

retrovirus, DNA virus, or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, inter alia encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of **peptide, the goal is to alleviate the sequestration of p53** by MDM2.

Detailed Description Text - DETX (18):

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to **human p53**. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

Detailed Description Text - DETX (27):

To determine whether the hMDM2 protein could bind to **human p53** protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping CDNA clones. The construct contained the sequence shown in FIG. 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Detailed Description Text - DETX (33):

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (FIG. 2, lanes 2 and 3). However, when in vitro translated **human p53** was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association in vitro (FIG. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (FIG. 2, lanes 8 and 9). When an in vitro translated mutant form of p53 (175.sup.his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

Detailed Description Text - DETX (59):

This assay was then applied to mapping the interaction domains of each protein. Full length CDNA **fragments encoding MDM2 or p53** were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

Detailed Description Text - DETX (60):

METHODS. Full length MDM2 CDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Kilenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K. W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D. M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive .beta.-galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. Thee MDM2 sequences of the two p53-independent clones are diagrammed in FIG. 6A. The MDM2 sequences of the remaining 15 **p53-dependent clones coded for peptides** ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of FIG. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Detailed Description Text - DETX (61):

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-**terminal** to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three **p53** sequences shown in FIG. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated **fragments**.

Detailed Description Text - DETX (62):

The resultant yeast colonies were examined for .beta.-galactosidase activity in situ. Of approximately 5000 clones containing MDM2 **fragments** fused to the lexA DNA binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of .beta.-galactosidase (about 5-fold less than the other fifteen clones) and .beta.-galactosidase expression was independent of **p53** expression (FIG. 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of

37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable .beta.-galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino **terminal** region of MDM2 (FIG. 6B). The .beta.-galactosidase activity of these clones was dependent on **p53** co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length **p53** contained MDM2 codons 1 to 118 (FIG. 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated **fragments** of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

Detailed Description Text - DETX (63):

In a converse set of experiments, yeast clones containing **fragments of p53** fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (FIG. 2C). The minimal overlap between these three **fragments** contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the **fragments** required 9-12 amino acids on either side of codons 13-41 for activity (FIG. 6C). To further test the idea that the amino **terminal** region of **p53** was required for MDM2 binding, we generated an additional yeast strain expressing the lexA-DNA binding domain fused to **p53** codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table 1, strain 8), as expected if the N-**terminus of p53** were required for the interaction.

Detailed Description Text - DETX (64):

Sequence analysis showed that all **p53 and MDM2 fragments** noted in FIG. 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in FIG. 6 expressed the relevant proteins at similar levels, as shown by Western blotting (FIG. 7).

Other Reference Publication - OREF (2):

Hinds, et al., "Mutant **p53 DNA Clones From Human** Colon Carcinomas Cooperate With Ras in Transforming Primary Rat Cells: A Comparsion of the "Hot Spot" Mutant Phenotypes", Cell Growth & Differentiation, 1:561-580 (1990).*

Other Reference Publication - OREF (5):

Oliner, et al., "Amplification of a Gene Encoding a **p53-Associated Protein in Human** Sarcomas", Nature, 358:80-83 (1992).*

Other Reference Publication - OREF (7):
    Leach, et al., "**p53 Mutation and MDMS Amplification in Human** Soft Tissue
Sarcomas", Cancer Research 53:2231-2234 (1993).*

US-PAT-NO: 6372249

DOCUMENT-IDENTIFIER: US 6372249 B1
See image for Certificate of Correction

TITLE: Senscent cell-derived inhibitors of DNA synthesis

DATE-ISSUED: April 16, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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Wilson; Deborah R.	Houston	TX	N/A	N/A
Zumstein; Louis A.	Houston	TX	N/A	N/A

APPL-NO: 08/ 327874

DATE FILED: October 24, 1994

PARENT-CASE:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of PCT US94/09700 (filed Aug. 26, 1994), which is a continuation-in-part of U.S. patent application Ser. No. 08/274,535 (filed Jul. 13, 1994) now abandoned, which is a continuation-in-part of U.S. patent application Ser. No. 08/229,420 (filed Apr. 15, 1994), now abandoned, which is a continuation-in-part of U.S. patent application Ser. No. 08/203,535 (filed Feb. 25, 1994), now abandoned, which is a continuation-in-part of U.S. patent application Ser. No. 08/153,564 (filed Nov. 17, 1993), now abandoned, which is a continuation-in-part of U.S. patent application Ser. No. 08/113,372 (filed Aug. 30, 1993), now abandoned, which is a continuation-in-part of U.S. patent application Ser. No. 07/970,462 (filed Nov. 2, 1992, and issued as U.S. Pat. No. 5,302,706 on Apr. 12, 1994); and divisional U.S. patent application Ser. No. 08/160,814 (filed Jan. 3, 1994,), now U.S. Pat. No. 5,424,400, and Ser. No. 08/268,439 (filed Jun. 30, 1994) now abandoned, all of which Applications are continuations-in-part of U.S. patent application Ser. No. 07/808,523 (filed Dec. 16, 1991, now abandoned).

US-CL-CURRENT: 424/450, 514/12 , 514/2 , 530/350

ABSTRACT:

The use of liposomal formulations, particularly formulations of positively charged and neutral lipids facilitates cellular uptake of SDI molecules. The transcription and/or expression of SDI-1-encoding nucleic acid molecules is facilitated by constructs that contain intervening untranslated regions.

7 Claims, 11 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 9

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Detailed Description Text - DETX (97):
    Since p53 is an inducer of SDI expression, it, or a nucleic acid encoding
p53, or biologically active fragments of either, may be provided to cells in
conjunction with an SDI molecule in order to obtain increased SDI expression.

Detailed Description Text - DETX (303):
    Therefore, to demonstrate the direct induction of SDI-1 by p53, the
above-described SDI-1 and antisense SDI-1 gene sequences were co-transfected
with a **p53 gene construct into normal human** fibroblasts. As expected, the
antisense construct was found to eliminate 80% of the inhibition of DNA
synthesis caused by SDI-1 alone. When 4 .mu.g of SDI-1 and increasing amounts
of p53 plasmids were co-transfected into MDAH 041 cells, the antisense SDI-1
was found to be capable of effectively counteracting the inhibition of DNA
synthesis caused by p53 alone. These findings are summarized in Table 7. This
finding verified the conclusion that one manner in which p53 causes inhibition
of DNA synthesis is by activating the expression of SDI-1 and that such
induction of SDI-1 is a requisite for part of the DNA synthesis-inhibitory
activity of p53. Such activation occurs, at least in part, by the
transcriptional activation of the SDI-1 gene. The expressed SDI-1 protein
acts, in part, by inhibiting the kinase activities of CDK/cyclin complexes and
can therefore act at multiple points in the cell cycle to block progression.
Loss of wild type p53 activity would lead to lack of expression of SDI-1 and
thereby result in inappropriate cell cycle progression.

Detailed Description Text - DETX (304):
    Mutations in the gene encoding **p53 protein are common in human** tumors with
approximately 50% of tumors expressing a mutant p53. This has led to the
conclusion that p53 acts as a negative growth regulator and is a tumor
suppressor gene. One aspect of the present invention concerns the recognition
of the molecular mechanism responsible for the anti-oncogene activity of p53.
SDI-1 has been found to be an inhibitor of cell cycle progression which acts at
least in part by inhibiting the kinase activities of cdk/cyclin complexes. As
such it can act at multiple points in the cell cycle to block progression.
Since p53 is required for transcriptional activation of SDI-1, inactivation of
this function could allow uncontrolled and inappropriate cell cycle
progression. This would allow cells to ignore the normal external singnals for
cell cycle stasis and permit proliferation in situ. Since SDI-1 is downstream
of p53, SDI-1 appears to be the effector of p53 action. Furthermore, mutations
have been found in SDI-1 which may contribute to altered cell proliferation in
cells without mutated p53.

Detailed Description Text - DETX (427):

Recent reports suggest that CPT may alter p34.sup.cdc2 /cyclin B complex regulation in HeLa cells (Tsao, Y. P. et al., Canc. Res. 52:1823-1829 (1992)) and induce wild type **p53** protein in ML-1 myeloid leukemia cells and in LNCaP prostatic adenocarcinoma cells (Nelson, W. G. et al., Molec. Cell. Biol. 14:1815-1823 (1994)). Significantly, both events appear to require active DNA synthesis. In this context, it has been reported that **p53** may activate wild type **p53-activated fragment** 1, SDI-1 (sometimes referred to as Waf-1) El-Deiry, W. S. et al., Cell 75:805-816 (1993)). As indicated above, SDI-1 plays a critical role in the regulation of cell growth in tumor and senescent cells by inhibiting cyclin-dependent **kinases** and by subsequently interrupting the cell division process. To expand these observations and to gain insight into the molecular mechanism of CPT-induced cytostasis, the expression of SDI-1 was evaluated in non-tumorigenic cells and the results were correlated with those from studies examining the effects of CPT on cell proliferation and metabolic activity, DNA synthesis, and perturbation in the cell cycle.

US-PAT-NO: 6265189

DOCUMENT-IDENTIFIER: US 6265189 B1

TITLE: Pox virus containing DNA encoding a cytokine and/or a tumor associated antigen

DATE-ISSUED: July 24, 2001

INVENTOR-INFORMATION:

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APPL-NO: 08/ 460736

DATE FILED: June 2, 1995

PARENT-CASE:

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a division of application Ser. No. 08/184,009, filed Jan. 19, 1994, U.S. Pat. No. 5,833,975. Application Ser. No. 08/184,009 is a continuation-in-part of application Ser. No. 08/007,115, filed Jan. 21, 1993, abandoned, incorporated herein by reference. Application Ser. No. 08/007,115 is a continuation-in-part of application Ser. No. 07/847,951, filed Mar. 6, 1992, abandoned, which in turn is a continuation-in-part of application Ser. No. 07/713,967, filed Jun. 11, 1991, abandoned, which in turn is a continuation-in-part of application Ser. No. 07/666,056, filed Mar. 7, 1991, abandoned; and, application Ser. No. 08/007,115 is also a continuation-in-part of application Ser. No. 07/805,567, filed Dec. 16, 1991, U.S. Pat. No. 5,378,457, which in turn is a continuation-in-part of application Ser. No. 07/638,080, filed Jan. 7, 1991, abandoned; and, application Ser. No. 08/007,115 is also a continuation-in-part of application Ser. No. 07/847,977, filed Mar. 3, 1992, abandoned; all of which are hereby incorporated by reference. Reference is also made to co-pending U.S. applications Ser. Nos. 07/715,921, abandoned, filed Jun. 14, 1991, 07/736,254, filed Jul. 26, 1991, abandoned, 07/776,867, filed Oct. 22, 1991, abandoned, and 07/820,077, filed Jan. 13, 1992, abandoned, all of which are hereby incorporated herein by reference.

US-CL-CURRENT: 435/70.1, 435/320.1 , 435/69.1 , 435/70.3

ABSTRACT:

Attenuated recombinant viruses containing DNA coding for a cytokine and/or a tumor associated antigen, as well as methods and compositions employing the viruses, are disclosed and claimed. The recombinant viruses can be NYVAC or

ALVAC recombinant viruses. The DNA can code for at least on of: human tumor necrosis factor; nuclear phosphoprotein **p53, wildtype or mutant; human** melanoma-associated antigen; IL-2; IFN.gamma.; IL-4; GNCSF; IL-12; B7; erb-B-2 and carcinoembryonic antigen. The recombinant viruses and gene products therefrom are useful for cancer therapy.

6 Claims, 46 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 33

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Abstract Text - ABTX (1):
   Attenuated recombinant viruses containing DNA coding for a cytokine and/or a tumor associated antigen, as well as methods and compositions employing the viruses, are disclosed and claimed. The recombinant viruses can be NYVAC or ALVAC recombinant viruses. The DNA can code for at least on of: human tumor necrosis factor; nuclear phosphoprotein **p53, wildtype or mutant; human** melanoma-associated antigen; IL-2; IFN.gamma.; IL-4; GNCSF; IL-12; B7; erb-B-2 and carcinoembryonic antigen. The recombinant viruses and gene products therefrom are useful for cancer therapy.

Drawing Description Text - DRTX (41):
   FIG. 39 shows the coding sequence for the **human p53** gene (SEQ ID NO:215).

Detailed Description Text - DETX (306):
   Generation of Poxvirus-based Recombinant Viruses Expressing Wildtype and Mutant Forms of the **Human p53** Gene Product

Detailed Description Text - DETX (307):
   Three plasmids, pS3wtXbaISP6/T3, p53-217Xbal, and **p53-238Xbal containing wildtype human p53** gene sequences, and two mutant forms of p53, respectively, were obtained from Dr. Jeffrey Marks (Duke University). The p53-217Xbal contains a p53 gene encoding a p53 product lacking codon 217 while p53-238Xbal encodes a p53 gene product with an cysteine to arginine substitution at amino acid 238. The sequence of the wildtype p53 cDNA and the deduced amino acid sequence was described previously (Lamb and Crawford, 1986; FIG. 3).

Detailed Description Text - DETX (309):
   PCR was also used to generate a 480 bp and 250 bp fragment from p53wtXbaISP6/T3. The 480 bp fragment was derived with oligonucleotides MM003 (SEQ ID NO:92) (5'-GTTTGTATCGTAATGGAGGAGCCGCAGTCAGATC-3') and MM008 (SEQ ID NO:93) (5'-CATTACGATACAAACTTAACGGATATCGCGACGCGTTCACACAGGGCAGGTCTTGGC-3')

This
fragment contains the 3' portion of the vaccinia virus H6 promoter sequences
and the 5' **portion f the p53** coding sequences through the SgrAI site. The 250
bp fragment was derived by amplification with oligonucleotides MM005 (SEQ ID
NO:94) (5'-TACTACCTCGAGCCCGGGATAAAAAACGCGTTCAGTCTGAGTCAGGCCC-3') and
MM007 (SEQ
ID NO:95)
(5'-GTGTGAACGCGTCGCGATATCCGTTAAGTTTGTATCGTAATGCAGCTGCGTGGGCGTGA
CTTC-3'). This PCR **fragment contains the 3' end of the p53** coding sequences
beginning at the StuI restriction site. The 480 bp and 250 bp PCR fragments
were generated such that the 5' end of the MM005/MM007-derived (SEQ ID
NO:94/95) fragment overlaps the 3' end of the MM003/MM008-derived (SEQ ID
NO:92/93) fragment.

Detailed Description Text - DETX (310):
   The 227 bp, 480 bp, and 250 bp PCR-derived **fragments** were pooled and fused
by PCR using oligonucleotides MM006 (SEQ ID NO:96)
(5'-ATCATCGGATCCCCCGGGTTCTTTATTCTATAC-3') and MM005 (SEQ ID NO:94). The
783 bp
fused PCR product contains the H6 promoter juxtaposed 5' to the 5' **portion of
the p53** coding sequence (through the SgrAI restriction site) followed by the
end of the **p53** coding sequence beginning at the StuI site. Following the end
of the **p53** coding sequence, a T.sub.5 NT sequence motif providing early
vaccinia transcription **termination** (Yuen and Moss, 1986) and a unique XhoI site
were added. It should be noted that the final H6-**p53** PCR fusion product (783
bp) does not contain the **p53** coding sequences between the SgrAI and StuI
restriction sites.

Detailed Description Text - DETX (312):
   Plasmids containing intact p53 gene (wildtype or mutant forms) juxtaposed 3'
to the H6 promoter were generated by first digesting pMM105 with SgrAI and
StuI. A 795 bp SgrAI/StuI **fragment was isolated from p53wtXbaISP6/T3 and
p53-238Xbal, while a 792 bp fragment was isolated from p53**-217Xbal. These
fragments were individually ligated to the SgrAI/StuI digested pMM105 plasmid
to yield pMM106, PMM108, and pMM107, respectively.

Detailed Description Text - DETX (315):
   ALVAC (CPpp) p53 insertion plasmids were engineered by excising the p53
expression cassettes from pMM106, pMM107, and pMM108 by digestion with BamHI
and XhoI and inserting them individually into BamHI/XhoI digested pNVQC5LSP-7.
The 1320 bp BamHI/XhoI **fragment containing the H6-p53** expression cassette from
pMM106 and pXM108 was inserted into pNVQC5LSP-7 to yield pMM110 and pMM112,
respectively, while the 1317 bp BamHI/XhoI fragment derived from pMM107 and
inserted into pNVQC5LSP-7 yielded pMM111.

Detailed Description Text - DETX (517):
   A **fragment containing the H6 pr m ted 5' end of the p53** gene fused to the 3'
end of the p53 gene was generated by several PCRs as described below.

Detailed Description Text - DETX (520):

PCRIII: Plasmid p11-4 was used as template with oligonucleotides MM084 (SEQ ID NO:212) 5' CAGAAGCTACTACTACTACTACCCACCTGCACAAGCGCC 3' and MM085 (SEQ ID NO:213) 5' AACTACTGTCCCGGGATAAAAATCAGTCTGAGTCAGGCCCCAC 3' to generate a 301 bp fragment. The 301 bp PCR-derived **fragment contains the 3' end of the p53** gene, and the 5' end overlaps the 3' end of the PCRII product. MM084 (SEQ ID NO:212) primes from position 916 of the murine p53 gene toward the 3' end. MM085 (SEQ ID NO:) primes from position 1173 toward the p53 gene 5' end. The three PCR products were pooled and primed with MM080 and MM085. The resultant 588 bp fragment contains a BamHI site followed by the H6 promoted 5' end of the p53 gene fused to the p53 gene 3' end followed by a SmaI site; the 5' end of the p53 gene ends at the XhoI site at position 37, and the 3' end starts at the SacII site at position 990 (FIG. 38). The 588 bp PCR-derived fragment was digested with BamHI and SmaI generating a 565 bp fragment which was inserted into BamHI/SmaI digested pNC5LSP5 (described below). The resultant plasmid, designated pMM136, was digested with KspI and XhoI to remove a 149 bp fragment, and the 953 bp KspI/XhoI fragment from p11-4 was inserted. The resultant plasmid, pMM148, contains the H6 promoted wild-type murine p53 in the ALVAC C5 insertion locus.

Detailed Description Text - DETX (528):

INSERTION OF MUTANT FORMS OF **HUMAN P53** INTO ALVAC AND NYVAC

Detailed Description Text - DETX (529):

Mutant forms of **Human p53** into ALVAC. FIG. 18 (Example 15) presented the sequence of the vaccinia H6 promoted **human wild type p53** gene cassette in an ALVAC-based recombinant, vCP207. In this example, to facilitate description of the mutant forms of the **human p53** gene being described, FIG. 39 (SEQ ID NO:215) presents only the coding sequence for the **human wild type p53** gene. The start codon is at position 1 and the stop codon is at position 1180.

Detailed Description Text - DETX (530):

Plasmid Cx22A, containing a mutant form of the **human p53** gene, was received from Arnold Levine (Princeton University, Princeton, N.J.). Relative to the wild type p53 sequence presented in FIG. 39, the G at nucleotide position 524 is substituted with an A, changing the arg amino acid at codon 175 of the wild type protein to a his amino acid in Cx22A.

Detailed Description Text - DETX (531):

Plasmid pMM110 (Example 15, FIG. 18) contains the vaccinia H6 promoted wildtype **human p53** gene in the ALVAC C5 insertion site. The **human p53** gene contains two PflmI sites. p53 coding sequences upstream from the first PflmI site and downstream from the second PflmI site are the same in pMM110 as in Cx22A. pMM110. was digested with PflmI to remove the 853 central base pairs of the p53 gene. The 853 bp PflmI fragment from Cx22A containing the base

change at position 524 was inserted. The resultant plasmid, pMM143, contains the H6 promoted mutant p53 gene.

Detailed Description Text - DETX (532):
   Recombination between donor plasmid pMM143 and ALVAC rescuing virus generated recombinant virus vCP270. vCP270 contains the mutant form of the **human p53** gene under the control of the vaccinia H6 promoter in the C5 locus.

Detailed Description Text - DETX (533):
   Plasmid pR4-2 containing a mutant form of the **human p53** gene was received from Arnold Levine (Princeton University, Princeton, N.J.). Relative to the wild type p53 sequence presented in FIG. 39, the G at nucleotide position 818 is substituted by an A, changing the arg codon at amino acid position 273 to a his codon in pR4-2.

Detailed Description Text - DETX (534):
   Plasmid pMM110 (Example 15, FIG. 18) contains the vaccinia H6 promoted **human wildtype p53** gene in the ALVAC C5 insertion site. p53 coding sequences upstream from the first Pflml site and p53 coding sequences downstream from the second Pflml site are the same in pMM110 as in pR4-2. pMM110 was digested with Pflml to remove the 853 central base pairs of the p53 gene. The 853 bp Pflml fragment from pR4-2 containing the base change at nucleotide position 818 was inserted. The resultant plasmid, pMM144, contains the H6 promoted mutant form of the **human p53** gene in the C5 insertion locus.

Detailed Description Text - DETX (535):
   Recombination between donor plasmid pMM144 and ALVAC rescuing virus generated recombinant virus vCP269. vCP269 contains the mutant form of the **human p53** gene under the control of the vaccinia H6 promoter in the C5 locus.

Detailed Description Text - DETX (536):
   Mutant forms of **Human p53** into NYVAC. Plasmid Cx22A, described above, contains a mutant form of the **human p53** gene, in which the G at nucleotide position 524 (FIG. 39) is substituted by an A, changing the arg codon at amino acid position 175 to a his codon in Cx22A.

Detailed Description Text - DETX (537):
   Plasmid pMM106 (Example 15) contains the vaccinia H6 promoted wild-type **human p53** gene in the NYVAC I4L insertion locus. p53 coding sequences upstream from the first Pflml site and p53 coding sequences downstream from the second Pflml site are the same in pMM106 as in Cx22A. pMM106 was digested with Pflml to remove the 853 central base pairs of the p53 gene. The 853 bp Pflml fragment from Cx22A containing the base change at position 524 was inserted. The resultant plasmid, pMM140, contains the H6 promoted mutant p53 gene.

Detailed Description Text - DETX (538):
   Recombination between donor plasmid pMM140 and NYVAC rescuing virus
generated recombinant virus vP1234. vP1234 contains the mutant form of the
human p53 gene under the control of the vaccinia H6 promoter in the I4L locus.

Detailed Description Text - DETX (539):
   Plasmid pR4-2, described above, contains a mutant form of the **human p53**
gene, in which the G at nucleotide position 818 (FIG. 39) is substituted by an
A, changing the arg codon at amino acid position 273 to a his codon in pR4-2.

Detailed Description Text - DETX (540):
   pMM106 (Example 15) contains the H6 promoted wild-type **human p53** gene in the
I4L locus. p53 coding sequences upstream from the first PflmI site and p53
coding sequences downstream from the second PflmI site are the same in pMM106
as in pR4-2. pMM106 was digested with PflmI to remove the 853 central base
pairs of the p53 gene. The 853 bp PflmI fragment from pR4-2 containing the
base change at position 818 was inserted. The resultant plasmid, pMM141,
contains the H6 promoted mutant p53 gene.

Detailed Description Text - DETX (541):
   Recombination between donor plasmid pMM141 and NYVAC rescuing virus
generated recombinant virus vP1233. vP1233 contains the mutant form of the
human p53 gene under the control of the vaccinia H6 promoter in the I4L locus.

Detailed Description Text - DETX (542):
   A listing of the wildtype and mutant forms of murine **p53 and the mutant
forms of human p53** present in ALVAC and NYVAC recombinants described in
Examples 31 and 32 is provided in Table 27.

Detailed Description Text - DETX (543):
   Immunoprecipitation. ALVAC and NYVAC based recombinants vP1101, vP1096,
vP1098, vCP207, vCP193, vCP191 (all described in Example 15; Table 22, as well
as ALVAC and NYVAC based recombinants VCP270, vCP269, vP1233, vP1234 described
in this Example, Table 27), contain wild type or mutant forms of the **human p53**
gene. All of these recombinant virus were assayed for expression of the **human
p53** gene using immunoprecipitation.

Detailed Description Text - DETX (544):
   Recombinant or parental virus were inoculated onto preformed monolayers of
tissue culture cells in the presence of radiolabelled .sup.35 S-methionine and
treated as previously described (Taylor et al., 1990). Immunoprecipitation
reactions were performed using a **human p53** specific monoclonal antibody 1801.
A protein of between 47 and 53 kDa was precipitated from cells infected with
any of the recombinant viruses, vP1101, vP1096, vP1098, vCP207, vCP193, vCP191,
vCP270, vCP269, vP1233, or vP1234, but not from uninfected cells or cells
infected with parental ALVAC or NYVAC virus.

Detailed Description Paragraph Table - DETL (45):

TABLE 27 Recombinant Virus Parent Virus Species Gene Insert vCP263 ALVAC murine w.t. p53 vCP267 ALVAC murine p53 (+3 aa) vCP270 ALVAC **human p53** (aa 175; R to H) vCP269 ALVAC **human p53** (aa 273; R to H) vP1234 NYVAC **human p53** (aa 175; R to H) vP1233 NYVAC **human p53** (aa 273; R to H)

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PARENT-CASE:

   This is a divisional application of Ser. No. 08/299,074, filed Sep. 1,
1994, now U.S. Pat. No. 5,955,263 which is a divisional of U.S. Ser. No.
07/860,758, filed Mar. 31, 1992, now U.S. Pat. No. 5,362,623 which is a
Continuation-In-Part application of U.S. Ser. No. 07/715,182, filed Jun. 14,
1991, now abandoned.

US-CL-CURRENT: 435/6, 536/23.1 , 536/24.33 , 536/24.5 , 536/25.3

ABSTRACT:

   Specific sequences in the human genome are the sites of strong binding of
wild-type p53 protein, but not mutant forms of the protein. These sequences
are used diagnostically to detect cells in which the amount of wild-type p53 is
diminished. The sequences can also be used to screen for agents which correct
for loss of wild-type p53 to DNA in cancer cells.

50 Claims, 35 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 28

---------- KWIC ---------

Brief Summary Text - BSTX (9):
   It is yet another object of the invention to provide a double-stranded DNA
fragment which contains a p53-specific DNA binding site.

Brief Summary Text - BSTX (13):

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment a method is provided for detecting the presence of wild-type p53 protein in a cell, comprising the steps of: contacting a **p53-specific-binding DNA fragment** with a cell lysate from a tissue of a human to bind the DNA **fragment to wild-type p53** present in the cell lysate; and detecting the binding of the **p53-specific-binding DNA fragment to wild-type p53**.

Brief Summary Text - BSTX (15):

In yet another embodiment a double-stranded DNA **fragment is provided which comprises a p53-specific-DNA binding site, wherein the fragment** comprises more than one monomer repeat of the sequence 5'-RRRCWWGYYY-3' (SEQ ID NO:3) and wherein the fragment is covalently attached to an insoluble polymeric support.

Brief Summary Text - BSTX (17):

In yet another embodiment of the invention a method is provided for identifying compounds which specifically bind to p53-specific DNA binding sequences, comprising the steps of: contacting a **p53-specific DNA binding fragment** immobilized on a solid support with a test compound to bind the test compound to the DNA fragment; and determining the amount of test compound which is bound to the DNA fragment.

Brief Summary Text - BSTX (18):

In even another embodiment of the invention a method is provided for identifying compounds which specifically bind to p53-specific-DNA binding sequences, comprising the steps of: contacting a **p53-binding DNA fragment** immobilized on a solid support with both a test compound and wild-type p53 protein to bind the wild-type **p53 protein to the DNA fragment; determining the amount of wild-type p53 protein which is bound to the DNA fragment, inhibition of binding of wild-type p53** protein by the test compound suggesting binding of the test compound to the p53-specific DNA binding sequences.

Brief Summary Text - BSTX (23):

In another embodiment of the invention a method is provided of diagnosing tumor-inducing or hyperplastia-inducing strains of human papilloma virus (HPV) comprising: contacting cells or cell extracts of patients suspected of being infected by HPV with a **p53-specific binding DNA fragment; and detecting the amount of wild-type p53 in said cells or cell extract which binds to said DNA fragment, absence of bound p53** indicating infection by strains of HPV which sequester p53.

Drawing Description Text - DRTX (2):

FIG. 1A. Screening for **fragments b und by p53** using an immunoprecipitation assay. Panel 1 contains the hFosAva2 clone; panel 2, 772 C.sub.BE ; panel 3,

Lambda 5R; panel 4, a pool of clones with inserts of randomly cloned human genomic sequences. 772 C.sub.BE and Lambda 5R contain Hinfl **fragments (259 and 190 bp, respectively) which b und p53** relatively strongly (arrowheads). "C"--control lane, containing 2% of the labelled DNA used in the binding reactions. "B"--bound DNA recovered from the immunoprecipitate. FIG. 1B. Tests for dependence on p53 and specific antibody. Cell lysates were produced by infection with vaccinia virus that did (+) or did not (-) contain an insert of wild-type p53 cDNA. Immunoprecipitation was performed with anti-p53 monoclonal antibodies (+) or normal mouse IgG (-).

Drawing Description Text - DRTX (3):

FIG. 2. Relative abilities of wild-type and mutant **p53 to precipitate fragment** A. "C"--control lanes, containing 2% of the labelled DNA used in the binding reaction, "B"--bound DNA recovered from the immunoprecipitate. FIG. 2A. Increasing quantities of wild-type and mutant 273.sup.his p53, affinity-purified from a baculovirus expression system, were used to precipitate labelled C.sub.BE fragments. FIG. 2B. Lysates from a vaccinia virus system (Vac) producing the wild-type (wt), mutant (175.sup.his), or no **p53 protein (-), were used to immunoprecipitate labelled C.sub.BE fragments**. Equivalent quantities of p53 were present in the wild-type and mutant p53 lysates, as assessed by Western blot. In the "Bac" lane, affinity-purified p53 produced in baculovirus-infected insect cells was used in place of the vaccinia-infected lysates.

Drawing Description Text - DRTX (5):

FIG. 4. Binding of various subfragments of **fragments A and B to p53** from vaccinia-infected cell lysates. FIG. 4A. Subfragments of fragment A (subclone 10d) were assayed by immunoprecipitation for their ability to bind wild-type p53 from vaccinia-infected cell lysates. Binding of at least 2% of the DNA added to the reaction was judged as a positive (+) result; lesser but significant binding was recorded as "+/-". Double Lines (=) denote fragment A sequences. Single lines (-) denote polylinker sequences of the vector, not originally present in fragment A (FIG. 1). Fragment 5mut1 had a G to T transversion at bp 120; 5mut2 had G to T transversions at bp 120 to 122. FIG. 4B. The fragment A (panels 1-4) and fragment B (panel 5) subfragments illustrated in FIG. 4A are labelled to the left of the bands. The "v" band in panel 4 corresponds to the 2.9 kb vector into which subfragment 6 was cloned. Subfragment 8 (panel 5) contained bp 104-238 of fragment B (see FIG. 3B). Control lanes (C) contained 2% of the labelled fragments used in the binding assays (B).

Drawing Description Text - DRTX (8):

FIG. 6B shows Southern blot analysis of transfected clonal lines. The exogenous **p53 gene was present on a 1.8 kb BamHI fragment**. The endogenous p53 gene gave rise to a 7.8 kb BamHI fragment. Other sized fragments presumably arose by rearrangements.

Drawing Description Text - DRTX (11):

FIG. 8. Isolation of **human genomic sequences which bound to p53**.

Drawing Description Text - DRTX (12):
    FIG. 8A. Experimental strategy used for isolation and analysis of human genomic DNA **fragments which bound to p53**.

Drawing Description Text - DRTX (14):
    Clones of amplified and selected (AS) DNA were tested for the presence of **p53-binding fragments** by IP. For each clone, the bound DNA is shown in the B lane, adjacent to a control (c) lane containing 2% of the total end-labeled DNA used in the binding assay. In this representative experiment, eight binding fragments were identified, representing six unique genomic fragments. The inserts from the clones in lanes labeled, 2, 3, 5, 9, 10, and 11 contained **p53-binding fragments,** while the other lanes contained none. The clones in lanes 2 and 5 each contained two binding fragments.

Drawing Description Text - DRTX (19):
    FIG. 11B. Comparison of the ability of wild-type and mutant p53 to bind to the consensus dimer. In vitro translated p53 proteins were tested for the ability to bind the consensus dimer by IP. Two percent of the total DNA used for binding is shown in lane 1. Lane 7 shows binding to baculovirus-produced **human wild-type p53** protein. Lanes 2 to 6 show binding of in vitro translated wild-type and mutant p53 proteins. The mutant p53 proteins contained changes at codon 143 (val to ala), 175 (arg to his), 248 (arg to trp), and 273 (arg to his).

Drawing Description Text - DRTX (24):
    FIG. 13A. Relative DNA-binding abilities of various length concatemers of a p53-binding sequence (PG.sub.n series), using an immunoprecipitation assay. Clones were cleaved by restriction endonucleases to extricate the concatemers, end-labelled, incubated with purified baculovirus-produced wild-type **human p53,** immunoprecipitated with anti-**p53 and protein A-Sepharose, and bound fragments** recovered and separated on a nondenaturing polyacrylamide gel. C, control lane, containing 2% of the labeled DNA used in the binding reactions. B, bound DNA recovered from the binding reactions.

Detailed Description Text - DETX (2):
    It is a finding of this invention that wild-type **p53 protein binds specific fragments** of human chromosomal DNA. Each of the fragments contains more than one monomer of the double-stranded motif 5'-RRRCWWGYYY-3' (SEQ ID NO: 3) separated by 0 to 13 bp. Some of these sequences are found near origins of replication of certain animal viruses and animal cells. See Jelinek et al, Proc. Natl. Acad. Sci. USA, vol. 77, pp. 1398-1402 (1980). Four mutant forms of **p53 protein which are commonly found in human** tumors do not have the ability to bind to these sequences. Thus, a function of p53 may be mediated by its ability to bind to specific DNA sequences in the human genome.

Detailed Description Text - DETX (5):

It has been found that **p53 will specifically bind t   other sequences in the human** genome with similar sequence motifs. Using a strategy coupling immunoprecipitation to "whole-genome PCR" (Kinzler, et al., Nucleic Acids Research, 17:3645-3653 (1989)), twenty **human DNA fragments that bind to p53** have been identified. Each of the fragments contain a sequence which conforms to a dimer of the double-stranded motif 5'-RRRCWWGYYY-3' (SEQ ID NO: 3), separated by 0 to 13 bp. These dimers directly mediate binding, as assessed by DNase I protection and methylation interference assays. The consensus dimers contain a striking symmetry, with four 5'-RRRCW-3' (SEQ ID NO: 39) units oriented in alternating directions. A synthetic monomer containing the 10 bp consensus sequence is insufficient for binding, while the combination of two or more monomers bind strongly to wt p53, but negligibly to p53 mutants. Thus, more than one monomer appears to be required for binding. The spacing between monomers may be from 0 to 40 nucleotides, although all natural binding sites isolated have spacings of less than 15 nucleotides. The symmetry of the four half-sites within the consensus dimers suggests that p53 interacts with DNA as a tetrameric protein. The twenty unique clones shown in FIG. 10 allow the identification of adjacent genes which may be regulated by p53 and may mediate its growth-suppressive action.

Detailed Description Text - DETX (8):

Based on the sequence information of the **p53 specific-DNA-binding fragments,** a number of diagnostic and therapeutic methods have been devised. According to one such method, cell lysates are tested for the presence or absence of wild-type p53 by virtue of its specific DNA binding ability. As it is known for various cancers and stages of cancers that one or both of the p53 alleles in tumor tissues can be mutant, testing for the presence or absence of wild-type p53 protein can provide diagnostic and prognostic information regarding a tumor and the patient. The cells to be tested are typically isolated from a tissue suspected of being neoplastic. Preferably the tissues are carefully prepared and isolated so that non-neoplastic tissues are not mixed with the neoplastic tissues, which can confound the analysis. Means for separating neoplastic tissues from non-neoplastic tissues are known in the art and include dissection of paraffin or cryostat sections, as well as use of flow cytometry. A cell lysate can be prepared from the tumor tissue according to any method known in the art. The cell lysate is then incubated with DNA **fragments which are known to bind the wild-type p53** protein, under conditions which are conducive to such DNA/protein interactions. Alternatively, a histological sample can be analyzed by incubation with DNA fragments, as described for cell lysates.

Detailed Description Text - DETX (11):

According to another embodiment of the invention, after incubation of **p53 with specific binding DNA fragments** all components of the cell lysate which do not bind to the DNA fragments are removed. This can be accomplished, among other ways, by employing DNA fragments which are attached to an insoluble polymeric support such as agarose, cellulose and the like. After binding, all non-binding components can be washed away, leaving p53 bound to the DNA/solid support. The p53 can be quantitated by any means known in the art. It can be determined using an immunological assay, such as an ELISA, RIA or Western

blotting.

Detailed Description Text - DETX (12):
    The diagnostic assay of the present invention has applicability not only
with regard to cancers which are known to involve mutation of **p53, but also
with regard to human** viruses such as human papilloma virus (HPV). HPV protein
E6 binds tightly to wild-type but not mutant p53. See Werness et al., Science,
248, 76-69 (1990). This tight binding is likely to block the interaction of
p53 with its specific DNA binding sequences. By testing cells or cell extracts
suspected of being infected with potentially tumor-inducing or
hyperplastia-inducing strains of HPV or possibly other viruses, infected cells
can be identified, because the E6 protein of the infected cells will have
sequestered the wild-type p53, rendering it unable to bind to its specific DNA
binding sequences. Such assays may be performed on cell extracts or on
histological specimens.

Detailed Description Text - DETX (13):
    According to the present invention a method is also provided of supplying
wild-type p53 function to a cell which carries mutant p53 alleles. The
wild-type p53 gene or a part of the gene may be introduced into the cell in a
vector such that the gene remains extra-chromosomal. In such a situation the
gene will be expressed by the cell from the extrachromosomal location. If the
mutant p53 genes present in the cell are expressed, then the wild-type **p53 gene
or gene portion** should be expressed to a higher level than that of the mutant
gene. This is because the mutant forms of the protein are thought to
oligomerize with wild-type forms of the protein. (Eliyahu et al., Oncogene,
vol. 3, p. 313, 1988.) If a gene **portion is introduced and expressed in a cell
carrying a mutant p53 allele, the gene portion should encode a part of the p53**
protein which is required for non-neoplastic growth of the cell. More
preferred is the situation where the wild-type p53 gene or a part of it is
introduced into the mutant cell in such a way that it recombines with the
endogenous mutant p53 gene present in the cell. Such recombination would
require a double recombination event which would result in the correction of
the p53 gene mutation. Vectors for introduction of genes both for
recombination and for extrachromosomal maintenance are known in the art and any
suitable vector may be used.

Detailed Description Text - DETX (15):
    According to the present invention compounds which have p53 activity are
those which specifically complex with a p53-specific DNA binding site.
Wild-type **p53 is one such compound, but portions of p53** which retain the
ability to bind to p53-specific binding sites may also be used.
Oligonucleotides and oligonucleotide containing nucleotide analogs are also
contemplated among those compounds which are able to complex with a
p53-specific DNA binding site. Although applicants do not wish to be bound by
any particular theory, it is believed that oligonucleotides bind
double-stranded DNA to form triplexes. Such triplexes have been shown to block
transcription of certain genes, as well as protect the DNA binding sites from
the action of enzymes such as DNA methylases. Although originally such
oligonucleotides were thought to require only or predominantly pyrimidines

(cytosine and thymine), purines have also successfully been incorporated into triplex forming oligonucleotides. Particular oligonucleotides which may be used include: nucleotides 140-162 of SEQ ID NO:2, nucleotides 128-158 of SEQ ID NO: 1, nucleotides 114-123 of SEQ ID NO: 1, or portions thereof having at least ten nucleotides.

Detailed Description Text - DETX (19):

Double-stranded DNA **fragments which comprise a p53**-specific DNA binding site and are attached to an insoluble polymeric support are also contemplated by this invention. The support may be agarose, cellulose, polycarbonate, polystyrene and the like. Such supported fragments may be used in screens to identify compounds which bind to p53-specific DNA binding sites. Similarly, such supported fragments may be used to perform diagnostic tests on cell lysates from suspected tumor tissues. They may also be used in assays used to screen potential chemotherapeutic agents, as discussed infra.

Detailed Description Text - DETX (20):

Although any method can be employed which utilizes the p53-specific DNA binding sites of the present invention, two particular methods are disclosed for screening for additional compounds that bind to p53-specific DNA binding sites. According to one method a test compound is incubated with a supported DNA fragment, as described above. The amount of test compound which binds to the supported DNA fragment is determined. This determination can be performed according to any means which is convenient. For example, the amount of a compound which can be removed after incubation with the supported fragment can be compared to the amount originally applied. Alternatively, the test compound can be labelled and the amount which binds to the supported fragment can be assayed directly. In order to render this screening method more specific, soluble DNA **fragments which do not contain the p53** DNA binding sequence can be added to the incubation mixture. The soluble **fragments would not have the ability to specifically bind to p53** wild-type protein.

Detailed Description Text - DETX (21):

According to another screening method for compounds to simulate the specific DNA binding activity of **p53, test compounds are incubated with supported DNA fragments** as described above. However, in this method wild-type p53 protein is also added to the incubation mixture. The amount of **p53 protein which binds to the DNA fragment** is measured using methods as described above. The amount of p53 protein bound is compared to the amount which binds in the absence of the test compound. Any diminution of p53 binding which results from the presence of the test compound is presumptively due to the competition of the test compound with **p53 for the specific DNA binding sites of the supported fragments**. Direct binding of the test compound to the binding site fragments can be confirmed using the assay described above.

Detailed Description Text - DETX (24):

Compounds which have p53-specific DNA-binding activity, including wild-type **p53 protein, polypeptides corresp nding t portions of wild-type p53** protein,

oligonucleotides and oligonucleotide containing nucleotide analogues, as well as other organic molecules can also be administered to humans and animals as a pharmaceutical and therapeutic composition. Effective amounts will be administered to cause neoplastic cells to become less aggressively neoplastic or even to stop the growth of the neoplastic cells entirely. Generally, such amounts will be in the range of 10 ng to 10 .mu.g per dose per person or other animal. The therapeutic compounds can be prepared in any conventional pharmaceutical excipient, such as physiological saline or other physiologically compatible aqueous buffer. Typically, the compounds will be administered by injection, either intravenous or intramuscular. However, other administration methods as are known in the art and may be used to administer the compounds of the present invention.

Detailed Description Text - DETX (30):
   In another embodiment of the invention, oligonucleotides can be isolated which restore to mutant p53 proteins the ability to bind to the consensus binding sequence or conforming sequences. Mutant p53 protein and random oligonucleotides are added to a solid support on which **p53-specific-binding DNA fragments** are immobilized. Oligonucleotides which bind to the solid support are recovered and analyzed. Those whose binding to the solid support is dependent on the presence of the mutant p53 protein are presumptively binding the support by binding to and restoring the conformation of the mutant protein.

Detailed Description Text - DETX (40):
   Each clone was digested with an appropriate restriction endonuclease, end-labelled with .sup.32 P, and incubated with p53 protein from a lysate of cells infected with a recombinant vaccinia virus expressing p53 protein. Labelled DNA **fragments which bound to p53** were then recovered by immunoprecipitation with monoclonal antibodies against p53. Of the more than 1400 restriction **fragments tested, only two bound reproducibly to p53** under the experimental conditions used: a 259 basepair Hinfl fragment (fragment A) of clone 772 C.sub.BE (Panel 2, FIG. 1A), and a 190 basepair Hinfl fragment (fragment B) of clone Lambda 5R (Panel 3, FIG. 1A); these fragments bound to a far greater extent than any of the other labelled fragments of larger or smaller size present in the same assay mixes.

Detailed Description Text - DETX (42):
   This example demonstrates that the immunoprecipitation of **fragment A is dependent on both p53** protein and anti-p53 antibodies.

Detailed Description Text - DETX (44):
   Lysates from cells infected with wild-type vaccinia virus (devoid of **p53) were not able to specifically immunoprecipitate fragment** A (FIG. 1B). Similarly, the detection of the precipitation of **fragment A was dependent on the presence of anti-p53** antibodies (FIG. 1B). The binding was evident in lysates prepared from either human HeLa cells or monkey BSC40 cells infected with vaccinia virus and expressing wild-type p53 (FIG. 1B).

Detailed Description Text - DETX (45):
   Affinity-purified baculovirus-produced wild-type p53 protein was substituted
for the vaccinia-infected cell lysates in the immunoprecipitation assay and
found to bind fragment A strongly (FIG. 2A). This suggested that the binding
to **fragment A was an intrinsic property of the p53** polypeptide and not
dependent on other factors present in the vaccinia virus-infected cell lysates.

Detailed Description Text - DETX (47):
   The example demonstrates that **p53 mutant proteins found in human** tumors fail
to bind to fragment A.

Detailed Description Text - DETX (48):
   Increasing quantities of wild-type and mutant 273.sup.his p53 protein,
affinity purified from a baculovirus expression system, were used to
immunoprecipitate labelled fragments from C.sub.BE. See FIG. 2A. The
proportion of **fragment A bound to wild-type p53** protein increased in tandem
with the amount of p53 added to the assay mixture. (FIG. 2A) In contrast,
fragment A did not specifically bind to a mutant form of p53 (273.sup.his)
protein even at the highest p53 protein concentration used. The 273.sup.his
mutation is the most common **p53 mutant identified in human** tumors. Another p53
mutant (175.sup.his) protein commonly found in human tumors also failed to bind
to fragment A (FIG. 1B).

Detailed Description Text - DETX (50):
   This example defines the particular sequences within **fragment A that allow
it to bind to wild-type p53** protein.

Detailed Description Text - DETX (52):
   One primer for each PCR was labelled with .sup.32 P at the 5' end with T4
polynucleotide **kinase** in a 5 .mu.l reaction, and the **kinase** inactivated at
70.degree. C. for 5 min. PCR contained 350 ng of each of the appropriate
primers and approximately 50 ng plasmid template in a 50 .mu.l reaction, using
25 cycles and the PCR conditions specified in Baker S J, et al., Cancer. Res.,
50:7717 (1990). The products were extracted with phenol and chloroform,
ethanol-precipitated, and dissolved in 3 mM Tris, 0.2 mM EDTA prior to binding.
Subfragment 1 contained bp 1 to 425 of subclone 10d of **fragment** A (FIG. 3A);
subfragments 1a, 1b, 1c, 1d, and 1e were generated by digestion of subfragment
1 with BamHI, MboI HindIII, HindIII, and BamHI, respectively, from **fragment** 1.
Subfragment 2, contained bp 283 to 425. Subfragment 3a was generated by
digestion of subfragment 3 (bp 106 to 294) with Hae III. Subfragment 4a was
produced from subfragment 4 (gp 1 to 141) by Hind III digestion. Subfragments
5a and 5b were products of the HaeIII digestion of subfragment 5 (bp 87 to
141). "Mutant" subfragments 5mut1 and 5 mut2 were produced using primers P3ml
(5'-GAAAGAAAAGGCAAGGCCAGGAAAGT-3') (SEQ ID NO: 34) and P3mut2
(5'-GAAAGAAAAGGCAAGGCCATTAAAGT-3') (SEQ ID NO: 35) and were identical to
subfragment 5 except for the positions underlined in the primers. Subfragment
6 contained bp 106 to 138, and the insert was excised by restriction with

HindIII and BamHI to generate 6a or with HindIII and EcoRI to generate 6b. Subfragment 3, including basepairs 106 to 294 (FIG. 4B, panel 2) bound well to **p53** as did subfragment 4, containing basepairs 1 to 141 (FIG. 4B, panel 3). This and similar assays done with additional subfragments (FIGS. 4A and 4B) localized the critical sequences to basepairs 106 to 141. This segment contained three repeats of the sequence TGCCT (SEQ ID NO: 36) (FIG. 3A). Digestion of subfragment 3 with HaeIII (cleaving between bp 125-126 and removing two of the TGCCT repeats) greatly reduced this binding (FIG. 4B, subfragment 3A, panel 2), suggesting that a critical sequence lay at or near this restriction site and that a single TGCCT (SEQ ID NO: 36) repeat was not sufficient for binding. Additional subfragments were tested (#5, bp 87 to 141, FIGS. 4A and 5B; #6, bp 106 to 138, FIGS. 4A and 4B, panel 4), and it was established that a 33 bp insert containing three TGCCT (SEQ ID NO: 36) repeats provided binding capability.

Detailed Description Text - DETX (54):
   This example demonstrates that certain G residues are critical for binding of **p53 to fragment** A.

Detailed Description Text - DETX (58):
   This example defines the region of **fragment B which is important for p53** binding.

Detailed Description Text - DETX (61):
   This example shows that expression of the wild-type **p53 gene in human** colorectal carcinoma cells dramatically inhibits their growth and that a mutant **p53 gene cloned from a human** colorectal carcinoma was incapable of exerting such inhibition.

Detailed Description Text - DETX (69):
   The conclusions made from the above experiments are dependent on the assumption that p53 protein was produced in the transfected cell lines. Clones containing exogenous mutant p53 sequences produced p53 mRNA at a concentration 1.5 to 3.5 times higher than that produced by the endogenous p53 gene (FIGS. 6A and 7A). Immunoblot analysis showed that there was a concomitant small increase in p53 protein expression in the transfectants (1.5- to 3-fold) compared to the untransfected cells. However, this increase was difficult to measure quantitatively, since these cells produced significant amounts of endogenous p53 protein that (unlike endogenous p53 mRNA) could not be distinguished from that produced by the vectors. To confirm that transfected **human cells expressed p53** protein from our constructs, we studied an additional colorectal carcinoma cell line (RKO). RKO cells were obtained through the generosity of M. Brattain. Although RKO cells did not contain a mutation within the susceptible p53 coding sequences, i.e., exons 5-9, they expressed low concentrations of p53 mRNA compared to normal colorectal mucosa or the other lines studied and did not produce detectable amounts of protein.

Detailed Description Text - DETX (70):

Results of colony formation assays in transfected RKO cells were similar to those in SW480 and SW837 cells. Colony formation by wild-type p53 gene transfectants occurred with a tenfold decrease in efficiency compared to the mutant p53 construct (Table 1). Immunocytochemical detection of p53 protein in transfected RKO cells was done as follows: approximately 5.times.10.sup.4 cells were cytocentrifuged onto polylysine-coated slides, fixed for 10 min in formalin, and permeabilized for 5 min in 0.5% Triton X-100. A mouse monoclonal antibody against **human p53** protein (Ab1801) in combination with the ABC immunoperoxidase system (Vector Laboratories), was used for immunocytochemical detection of p53 protein (Banks, et al., Eur. J. Biochem. 159, 529 (1986)). Ten to 20 randomly selected microscopic fields were analyzed per slide. These observations are consistent with the greater stability of mutant compared to wild-type p53 protein noted previously (C. A. Finlay et al., Mol. Cell Biol. 8, 531 (1988)). However, transient mRNA expression was also significantly lower in the SN3 transfectants compared to the SCX3 transfectants at 48 and 96 hours, supporting the idea that RKO cells expressing wild-type p53 were at a selective disadvantage compared to those producing mutant p53 products.

Detailed Description Text - DETX (76):
   This example demonstrates the identification of human genomic **fragments that can bond to wt p53** protein in vitro.

Detailed Description Text - DETX (78):
   Following the outline in FIG. 8A., we tested the inserts of 530 clones for binding to p53. Restriction fragments of the clones were end-labeled and incubated with purified **human wt p53** protein produced in baculovirus-infected cells.

Detailed Description Text - DETX (79):
   Whole-genome PCR was performed as previously described, except that only one oligonucleotide (5'-GAGTAGAATTCTAATATCTC-3') (SEQ ID NO: 37) was used for amplification (Kinzler, et al. (1989), Nucleic Acids Research, 17:3645-3653, and Kinzler, et al. (1990), Molec. Cell. Biol., 10:634-642). Two hundred ng of "catch"-linked human genomic DNA were incubated with 100 ng of baculovirus-produced **human wt p53** purified as described (Friedman, et al. (1990), Proc. Natl. Acad. Sci. U.S.A., 87:9275-9279), and immunoprecipitated as described below. After 4 rounds of IP and PCR, the AS DNA was cleaved with Eco RI and cloned into either the vector Lambda Zap II or pBluescript II SK+ (Stratagene). Individual clones were picked at random and tested for p53 binding. In panel B, cloned plasmid DNA samples were cleaved with Eco RI and end-labeled by Klenow fill-in. For IP (McKay, et al. (1981), J. Mol. Biol, 145:471-479), ten ng of DNA were incubated with 100 ng of baculovirus-produced **human wt p53** and 100 ng of poly dl-dC at 4.degree. C. for 30 minutes in 100 .mu.l of "DNA-binding buffer" containing 100 mM NaCl, 20 mM Tris, pH 7.0, 10% glycerol, 1% NP40, and 5 mM DTT. DNA **fragments bound to p53** were complexed to antibodies by the addition of 8 .mu.l containing 400 ng each of anti-p53 antibodies pAb421 and pAb1801, both obtained from Oncogene Science, and incubated for 30 minutes at 4.degree. C. The DNA-binding buffer containing 1.5 mg protein A precipitated following the addition of 26 .mu.l of DNA-binding buffer containing 1.5 mg protein A Sepharose and 10 .mu.g of poly dl-dC and

mixing at 4.degree. C. for 30 minutes. After removal of the supernatant, the immunoprecipitate was washed twice with 1 ml of DNA-binding buffer. Bound DNA was purified by treatment with SDS and proteinase K at 48.degree. C. for 30 minutes, extracted with phenol and chloroform, precipitated with ethanol, separated by electrophoresis on a 10% nondenaturing polyacrylamide gel, and autoradiographed.

Detailed Description Text - DETX (80):

Twenty-three of the clones were found to contain **fragments that bound to p53**. Examples of the IP experiments are shown in FIG. 8B. Clone S61 (lanes 11B,C) contains a single genomic **fragment of 202 bp which bound to p53**. Clone N2 contained five **fragments, only one of which (357 bp) bound to p53** (lanes 10B,C). Other examples of **p53-binding fragments** were obtained, and each of these was subcloned for further analysis. In contrast, we found that none of over 1000 clones containing unselected human DNA inserts of similar size bound to p53 using the IP assay. Thus, the whole-genome PCR procedure significantly enriched for p53-binding sequences.

Detailed Description Text - DETX (82):

This example demonstrates the localization of **p53 contacts with bound DNA fragments**.

Detailed Description Text - DETX (83):

Localization of the regions bound by p53 was obtained by DP or MI assays using the subcloned DNA fragments as probes. For MI, the **fragments were methylated at G residues and bound to p53** (FIG. 9). Methylation of G residues critical for p53 binding resulted in interference with IP. For example, methylation at nucleotides, 217, 22, 227 to 229, and 233 of the 248 bp insert from clone 11B3 completely interfered with the binding of this **fragment to p53** (FIG. 9, footprint 2). When the opposite strand was analyzed, interference was observed at the G residues corresponding to nucleotides 219, 223, 224, 230, 235, and 236 (FIG. 9, footprint 1). For DP, labelled DNA fragments were first subject to IP, then incubated with various amounts of DNase I. For clone N22, p53 binding provided protection against DNase I cleavage at residues 187 to 211 (FIG. 9, footprint 9). MI showed interference by G residues only within the region protected by DNase I (FIG. 9, footprint 10). Other examples of DP and MI mapping are shown in FIG. 9. **p53-binding DNA fragments** were subcloned and labeled on one end, gel-purified and subjected to DP or MI mapping. For MI, 10 ng of DNA were incubated in 200 .mu.l of 50 mM Na-cacodylate, 1 mM EDTA, pH 8.0 and 5 .mu.l of 10% dimethylsulfate/90% ethanol for 5 minutes at 20.degree. C. to methylate G residues. Fifty .mu.l containing 1.5 m Na-acetate, 1 M .beta.-mercaptoethanol and 60 .mu.g of glycogen were added. The mixture was ethanol-precipitated, washed, and resuspended in 5 .mu.l of 3 mM Tris, 0.2 mM EDTA, pH 7.5, and allowed to bind to wild-type p53 as described in the legend to FIG. 1. After IP and DNA purification, the samples were incubated with 100 .mu.l of 1 M piperidine at 90.degree. C. for 30 minutes. The samples were then dried under vacuum and separated electrophoretically on a 6% polyacrylamide sequencing gel. The control DNA samples were carried through all incubations except no p53 was added. For these control samples, the

protein A Sepharose pellets were treated with SDS and proteinase K without removal of the supernatants (which contained the labeled DNA in the absence of p53).

Detailed Description Text - DETX (84):
   For DP assays, end-labeled DNA fragments were immunoprecipitated as described in the legend to FIG. 8. The protein A Sepharose pellets were incubated for two minutes at 25.degree. C. with 200 ng DNase I in 5 mM MgCl.sub.2. After purification of the DNA, as described above, samples were separated by electrophoresis on sequencing gels and loaded as described above for MI. MI was performed on all 18 genomic DNA **fragments which bound to p53**. DP assays were performed on 13 fragments and the regions of protection uniformly coincided with those indicated by the MI assays.

Detailed Description Text - DETX (94):
   This example demonstrates that intact **p53 can activate expression in human** cells.

Detailed Description Text - DETX (95):
   We first made reporter plasmids (PG.sub.n -CAT series) containing part of the polyomavirus early promoter and the CAT gene located downstream of DNA sequences which could bind to **p53** in vitro (FIG. 8). For the CAT reporters, concatemers of the **p53**-binding region of C.sub.BE were formed by ligation of complementary oligonucleotides, ligated into the EcoRV site of pBluescript II SK+ (Stratagene) to form the PG.sub.n and MG.sub.n series. The BglII-BamHI **fragment** of pPyOICAT (Murakami, et al. (1990) Oncogene, 5:5), containing the polyomavirus early promoter and the CAT gene coding region, was ligated into the BamHI site of the PG.sub.n and MG.sub.n series clones to form the PG.sub.n -CAT and MG.sub.n -CAT series, and the orientation of the inserts characterized by restriction enzyme analysis. The PG.sub.9 -MG.sub.n -CAT and PG.sub.13 -MG.sub.n -CAT series were formed by excising the HindIII-SalI **fragments** of PG.sub.9 -CAT and PG.sub.13 -CAT, blunt-ending, attaching XbaI linkers, and ligating into the XbaI site of the MG.sub.n -CAT series plasmids (where n=1, 5, 10, and 15). For the yeast .beta.-galactosidase reporter plasmids, PG and MG sequences were ligated as SalI-SmaI **fragments** to the SalI and filled-in XhoI sites of pCZ (Buchanan, et al. (1988), Mol. Cell Biol., 8:50806). The construction of the **p53**-wt expression construct has been described (Baker, et al. (1990), Science, 249:912); the mutant expression plasmids were constructed similarly from the previously described cDNA plasmids (Nigro, et al. (1989), Nature, 342:705, and Kern, et al. (1991), Oncogene, 6:131), or in the case of the engineered **phosphorylation** site mutants, by in vitro mutagenesis (Altered Sites, Promega) with verification by sequencing. The construction of the yeast **p53** expression vectors based on pRS314 has been described (Nigro, et al., Mol. Cell Biol. (in press)).

Detailed Description Text - DETX (96):
   For the p53 binding sequences, we used a series of concatemers of the oligonucleotide PG (5'-CCTGCCTGGACTTGCCTGG-3') (SEQ ID NO: 40). This contained

the binding region of plasmid C.sub.BE, previously shown to bind p53 in vitro. The reporter and an expression vector coding for the intact **human wild-type protein (p53**-wt) (FIG. 12B), were transfected together into the human colorectal cancer cell line HCT 116. This line makes low amounts of apparently wild-type p53 protein.

Claims Text - CLTX (7):
   7. An isolated and purified double-stranded DNA **fragment which comprises a p53-specific DNA binding site, wherein the fragment** comprises more than two monomers of the sequence TGCCT, and wherein the fragment is covalently attached to an insoluble polymeric support.

Other Reference Publication - OREF (7):
   Mercer et al., "Negative Growth Regulation in a Gliblastoma Tumor Cell Line That Conditionally Expresses **Human Wild-Type p53**", Proc. Natl. Acad. Sci. USA 87:6166-6170 (1990).

Other Reference Publication - OREF (12):
   Romano et al., "Identification and Characterization of a **p53 Gene Mutation in a Human** Osteosarcoma Cell Line", Oncogene 4:1483-1488 (1989).

Other Reference Publication - OREF (14):
   Nigro et al., "Mutations in the **p53 Gene Occur in Diverse Human** Tumor Types", Nature 342:705-708 (1989).

US-PAT-NO: 6069134

DOCUMENT-IDENTIFIER: US 6069134 A
See image for Certificate of Correction

TITLE: Methods and compositions comprising DNA damaging agents
                and p53

DATE-ISSUED: May 30, 2000

INVENTOR-INFORMATION:

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APPL-NO: 08/ 953290

DATE FILED: October 17, 1997

PARENT-CASE:

   This is a divisional application of Ser. No. 08/233,002 filed Apr. 25,
1994, now U.S. Pat. No. 5,747,469, issued May 5, 1998.

US-CL-CURRENT: 514/44, 424/93.21 , 435/320.1 , 435/325 , 435/455 , 435/458
            , 435/69.1

ABSTRACT:

   The present invention relates to the use of tumor suppressor genes in
combination with a DNA damaging agent or factor for use in killing cells, and
in particular cancerous cells. A tumor suppressor gene, p53, was delivered via
a recombinant adenovirus-mediated gene transfer both in vitro and in vivo, in
combination with a chemotherapeutic agent. Treated cells underwent apoptosis
with specific DNA fragmentation. Direct injection of the p53-adenovirus
construct into tumors subcutaneously, followed by intraperitoneal
administration of a DNA damaging agent, cisplatin, induced massive apoptotic
destruction of the tumors. The invention also provides for the clinical
application of a regimen combining gene replacement using replication-deficient
wild-type p53 adenovirus and DNA-damaging drugs for treatment of human cancer.

69 Claims, 43 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 21

Drawing Description Text - DRTX (5):

FIG. 2B. Agarose gel analysis of PCR products. Two pairs of primers that define 1.4-kb (**p53) and 0.86-kb (Ad5) DNA fragments** were used in each reaction. DNA templates used in each reaction were pEC53 plasmid (lane 1), Ad5/RSV/GL2 DNA (lane 2), no DNA (lane 3), and Ad5CMV-p53 DNA (lane 4). The lane labeled (M) corresponds to molecular weight markers.

Detailed Description Text - DETX (11):

Casey and colleagues have reported that transfection of DNA encoding wild-type **p53 into two human** breast cancer cell lines restores growth suppression control in such cells (Casey et al., 1991). A similar effect has also been demonstrated on transfection of wild-type, but not mutant, **p53 into human** lung cancer cell lines (Takahasi et al., 1992). The p53 appears dominant over the mutant gene and will select against proliferation when transfected into cells with the mutant gene. Normal expression of the transfected p53 does not affect the growth of cells with endogenous p53. Thus, such constructs might be taken up by normal cells without adverse effects.

Detailed Description Text - DETX (29):

The present invention provides cancer gene therapy with a new and more effective tumor suppressor vector. This recombinant virus exploits the advantages of adenoviral vectors, such as high titer, broad target range, efficient transduction, and non-integration in target cells. In one embodiment of the invention, a replication-defective, helper-independent adenovirus is created that expresses wild type p53 (Ad5CMV-**p53) under the control of the human** cytomegalovirus promoter.

Detailed Description Text - DETX (32):

The design and propagation of the preferred p53 adenovirus is diagramed in FIG. 1. In connection with this, an improved protocol has been developed for propagating and identifying recombinant adenovirus (discussed below). After identification, the p53 recombinant adenovirus was structurally confirmed by the PCR analysis, as indicated in FIG. 2. After isolation and confirmation of its structure, the **p53 adenovirus was used to infect human** lung cancer cell line H358, which has a homozygous p53 gene deletion. Western blots showed that the exogenous p53 protein was expressed at a high level (FIG. 4 and FIG. 5) and peaked at day 3 after infection (FIG. 6).

Detailed Description Text - DETX (33):

It was also shown in a p53 point mutation cell line H322 that the mutant p53 was down regulated by the expression of the exogenous p53. As an experimental control, a virion (Ad5/RSV/GL2) that had a structural similarity to that of Ad5CMV-p53 was used. This virion contained a luciferase CDNA driven by Rous sarcoma virus LTR promoter in the expression cassette of the virion. Neither

p53 expression nor change in actin expression was detected in cells infected by the virion Ad5/RSV/GL2. Growth of the H358 cells infected with Ad5CMV-p53 was greatly inhibited in contrast to that of noninfected cells or the cells infected with the control virion (FIG. 7A). Growth of H322 cells was also greatly inhibited by the **p53 virion (FIG. 7B), while that f human** lung cancer H460 cells containing wild-type p53 was less affected (FIG. 7C).

Detailed Description Text - DETX (35):
   Tests in nude mice demonstrated that tumorigenicity of the Ad5CMV-p53-treated H358 cells was greatly inhibited. In a mouse model of orthotopic human lung cancer, the tumorigenic H226Br cells, with a point mutation in p53, were inoculated intratracheally 3 days prior to the virus treatment. Intratracheal instillation of Ad5CMV-p53 prevented tumor formation in this model system suggesting that the modified adenovirus is an efficient vector for mediating transfer and expression of tumor suppressor genes in **human cancer cells and that the Ad5CMV-p53** virus may be further developed into a therapeutic agent for use in cancer gene therapy.

Detailed Description Text - DETX (36):
   Ad5CMV-p53 mediated a high level of expression of the **p53 gene in human** lung cancer cells as demonstrated by Western blot analysis. Exogenous p53 protein was approximately 14 times more abundant than the endogenous wild-type p53 in H460 cells and about two to four times more abundant than the .beta.-actin internal control in H358 cells. The high level of expression may be attributed to (1) highly efficient gene transfer, (2) strong CMV promoter driving the p53 CDNA, and (3) adenoviral E1 enhancer enhancing the p53 CDNA transcription. The duration of p53 expression after infection was more than 15 days in H358 cells. However, there was a rapid decrease in expression after postinfection day 5. PCR analysis of the DNA samples from the infected H358 cells showed a decrease of the viral DNA level with the decreased protein level, indicating the loss of viral DNA during the continuous growth of cancer cells in vitro.

Detailed Description Text - DETX (85):   .
   Ad5CMV-p53-Directed **p53 Gene Expression in Human** Lung Cancer Cells

Detailed Description Text - DETX (86):
   This example describes the use of recombinant **p53 adenovirus to infect human** lung cancer cells with a homozygous p53 gene deletion. The results show that growth of these cells and expression of mutant p53 was suppressed, indicating the potential of the Ad5CMV-p53 virion as a useful agent for control of metastatic cells.

Detailed Description Text - DETX (90):
   Western blotting analysis was performed on total cell lysates prepared by lysing monolayer cells in dishes with SDS-PAGE sample buffer (0.5 ml per 60-mm dish) after rinsing the cells with phosphate-buffered saline (PBS). For SDS-PAGE analysis lanes were loaded with cell lysates equivalent to $5.times.10.sup.4$ cells (10-15 ml). The proteins in the gel were transferred to

Hybond.TM.-ECL membrane (Amersham, Arlington Heights, Ill.). The membranes were blocked with 0.5% dry milk in PBS and probed with the primary antibodies: mouse anti-**human p53** monoclonal antibody PAb 1801 and mouse anti-human .beta.-actin monoclonal antibody (Amersham), washed and probed with the secondary antibody: horseradish peroxidase-conjugated rabbit anti-mouse IgG (Pierce Chemical Co., Rockford, Ill.). The membranes were developed according to the Amersham's enhanced chemiluminescence protocol. Relative quantities of the exogenous p53 expressed were determined by densitometer (Molecular Dynamics Inc., Sunnyvale, Calif.).

Detailed Description Text - DETX (94):

Growth of the H358 cells infected with Ad5CMV-p53 was greatly inhibited in contrast to that of noninfected cells or the cells infected with the control virion (FIG. 7A). Growth of H322 cells was also greatly inhibited by the **p53 virion (FIG. 7B), while that of human** lung cancer H460 cells containing wild type p53 was affected to a lesser degree (FIG. 7C). Growth of the Ad5CMV-p53 virus-infected H358 cells was inhibited 79%, whereas that of noninfected cells or the cells infected with the control virus were not inhibited. Growth of cell line H322, which has a point mutation in p53, was inhibited 72% by Ad5CMV-p53, while that of cell line H460 containing wild-type p53 was less affected (28% inhibition).

Detailed Description Text - DETX (116):

The construction and identification of a recombinant adenovirus vector that contains the cDNA that encodes **human wt-p53** (Ad-p53) or luciferase (Ad-Luc) were previously reported (Zhang, et al., 1993). Briefly, the **p53 expression cassette that contains human cytomegalovirus promoter, wt-p53** cDNA, and SV40 early polyadenylation signal, was inserted between the XbaI and ClaI sites of pXCJL.1. The p53 shuttle vector and the recombinant plasmid pJM17 were cotransfected into 293 cells (Ad5-transformed human embryonic kidney cell line) by a liposome-mediated technique. The culture supernatant of 293 cells showing the complete cytopathic effect was collected and used for subsequent infections. The control Ad-Luc virus was generated in a similar manner. Ad-p53 and Ad-Luc viruses were propagated in 293 cells. The presence of replication competent virus was excluded by HeLa cell assays. The viral titers were determined by plaque assays (Graham, et al., 1991).

Detailed Description Text - DETX (121):

H358 cells were transduced in vitro with the **human wt-p53** cDNA by exposure to Ad-p53. Western blot analysis showed a high level of wt-p53 protein expression as early as 24 hours after infection with Ad-p53, but no wt-p53 was detected in parental (uninfected) cells or control cells infected with Ad-Luc (data not shown). Concurrent immunohistochemical evaluation demonstrated detectable wt-p53 protein in more than 80% of infected cells, suggesting that the transfer and expression of p53 by AD-p53 was highly efficient (data not shown).

Detailed Description Text - DETX (123):

An internucleosomal DNA ladder indicative of DNA fragmentation was evident

in cells expressing wt-**p53** after 24 hours of exposure to CDDP; parental and Ad-Luc-infected cells, however, did not show DNA fragmentation (FIG. 11A). **Terminal** deoxynucleotidyl transferase (TdT)-mediated 2'-deoxyuridine-5'-triphosphate (dUTP)-biotin nick end labeling, which detects DNA fragmentation characteristic of apoptosis in situ, showed many apoptotic cells in Ad-**p53**-infected cells treated with CDDP for 24 hours as shown in FIG. 11G which demonstrates darkly staining nuclei and nuclear **fragments** not present in FIGS. 11B-F.

Detailed Description Text - DETX (151):
   Cai, D. W., Mukhopadhyay, T., Liu, T., Fujiwara, T., and Roth, J. A. Stable expression of the wild-type **p53 gene in human** lung cancer cells after retrovirus-mediated gene transfer. Human Gene Ther, 4: 617-624, 1993.

Detailed Description Text - DETX (160):
   Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Cai, D. W., Owen-Schaub, L. B., and Roth, J. A. A retroviral wild-type **p53 expression vector penetrates human** lung cancer spheroids and inhibits growth by inducing apoptosis. Cancer Res, 53: 4129-4133, 1993.

Detailed Description Text - DETX (170):
   Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. (1991). **p53 mutations in human** cancers. Science 253:49-53.

Detailed Description Text - DETX (195):
   Takahashi, T., Carbone, D., Takahashi, T., Nau, M. M., Hida, T., Linnoila, I., Ueda, R., and Minna, J. D. (1992). Wild-type but not mutant **p53 suppresses the growth of human** lung cancer cells bearing multiple genetic lesions. 1992. Cancer Res. 52:2340-2342.

Detailed Description Text - DETX (207):
   Zhang, W. W., Fang, X., Mazur, W., French, B. A., Georges, R. N., and Roth, J. A. High-efficiency gene transfer and high-level expression of wild-type **p53 in human** lung cancer cells mediated by recombinant adenovirus. Cancer Gene Therapy, 1993.

Other Reference Publication - OREF (14):
   Chen et al., "Expression of Wild-Type **p53 in Human** A673 Cells Suppresses Tumorigenicity but Not Growth Rate," Oncogene, 6:1799-1805, 1991.

Other Reference Publication - OREF (15):
   Chen et al., "Genetic Mechanisms of Tumor Suppression by the **Human p53** Gene," Science, 250:1576-1580, 1990.

Other Reference Publication - OREF (16):
   Cheng et al., "Suppression of Acute Lymphoblastic Leukemia by the **Human Wild-Typ p53** Gene", Cancer Research, 52:222-226, Jan. 1, 1992.

Other Reference Publication - OREF (42):
   Fujiwara et al., "A Retroviral Wild-type **p53 Expression Vector Penetrates Human** Lung Cancer Spheriods and Inhibits Growth by Inducing Apoptosis," Cancer Research, 53:4129-4133, 1993.

Other Reference Publication - OREF (93):
   Mercer et al., "Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses **human wild-type p53**", Proc. Natl. Acad. Sci. USA, 87:6166-6170, Aug. 1990.

Other Reference Publication - OREF (103):
   Nigro et al., "Mutations in the **p53 gene occur in diverse human** tumour types", Nature, 342:705-708, Dec. 7, 1989.

Other Reference Publication - OREF (116):
   Rau et al., "Response of **p53 to treatment with actinomycin D in human** mammary carcinoma cell lines," Journal of Cancer Research and Clinical Oncology, 120:R108, 1994.

Other Reference Publication - OREF (123):
   Shaw et al., "Induction of apoptosis by wild-type **p53 in human** colon tumor-derived cell line," Proc. Natl. Acad. Sci. USA, 89(10):4495-4499, 1992.

Other Reference Publication - OREF (124):
   Shay et al., "A Role for Both RB and **p53 in the Regulation of Human** Cellular Senescence", Experimental Cell Research, 196:33-39, 1991.

Other Reference Publication - OREF (135):
   Takahashi et al., "Wild-Type but Not Mutant **p53 Suppresses the Growth of Human** Lung Cancer Cells Bearing Multiple Genetic Lesions," Cancer Research, 52:2340-2343, 1992.

Other Reference Publication - OREF (140):
   Ullrich et al., "**Human wild-type p53** adopts a unique conformational and phosphorylation state in vivo during growth arrest of glioblastoma cells", Oncogene, 7:1635-1643, 1992.

US-PAT-NO: 5955263

DOCUMENT-IDENTIFIER: US 5955263 A

TITLE: Sequence specific DNA binding by p53

DATE-ISSUED: September 21, 1999

INVENTOR-INFORMATION:

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APPL-NO: 08/ 299074

DATE FILED: September 1, 1994

PARENT-CASE:

This application is a division of application Ser. No. 07/860,758, filed Mar. 31, 1992 now issued as U.S. Pat. No. 5,362,623, which is a continuation-in-part of application Ser. No. 07/715,182, filed Jun. 14, 1991 now abandoned.

US-CL-CURRENT: 435/6, 435/7.23 , 436/63 , 436/64 , 536/23.1 , 536/24.1

ABSTRACT:

Specific sequences in the human genome are the sites of strong binding of wild-type p53 protein, but not mutant forms of the protein. These sequences are used diagnostically to detect cells in which the amount of wild-type p53 is diminished. The sequences can also be used to screen for agents which correct for loss of wild-type p53 to DNA in cancer cells.

24 Claims, 35 Drawing figures

Exemplary Claim Number: 1,23

Number of Drawing Sheets: 28

---------- KWIC ---------

Brief Summary Text - BSTX (9):
It is yet another object of the invention to provide a double-stranded DNA **fragment which contains a p53**-specific DNA binding site.

Brief Summary Text - BSTX (13):

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment a method is provided for detecting the presence of wild-type p53 protein in a cell, comprising the steps of: contacting a **p53-specific binding DNA fragment** with a cell lysate from a tissue of a human to bind the DNA **fragment to wild-type p53** present in the cell lysate; and detecting the binding of the **p53-specific-binding DNA fragment to wild-type p53**.

Brief Summary Text - BSTX (15):

In yet another embodiment a double-stranded DNA **fragment is provided which comprises a p53-specific-DNA binding site, wherein the fragment** comprises more than one monomer repeat of the sequence 5'-RRRCWWGYYY-3' (SEQ ID NO:3) and wherein the fragment is covalently attached to an insoluble polymeric support.

Brief Summary Text - BSTX (17):

In yet another embodiment of the invention a method is provided for identifying compounds which specifically bind to p53-specific DNA binding sequences, comprising the steps of: contacting a **p53-specific DNA binding fragment** immobilized on a solid support with a test compound to bind the test compound to the DNA fragment; and determining the amount of test compound which is bound to the DNA fragment.

Brief Summary Text - BSTX (18):

In even another embodiment of the invention a method is provided for identifying compounds which specifically bind to p53-specific-DNA binding sequences, comprising the steps of: contacting a **p53-binding DNA fragment** immobilized on a solid support with both a test compound and wild-type p53 protein to bind the wild-type **p53 protein to the DNA fragment; determining the amount of wild-type p53 protein which is bound to the DNA fragment, inhibition of binding of wild-type p53** protein by the test compound suggesting binding of the test compound to the p53-specific DNA binding sequences.

Brief Summary Text - BSTX (23):

In another embodiment of the invention a method is provided of diagnosing tumor-inducing or hyperplastia-inducing strains of human papilloma virus (HPV) comprising: contacting cells or cell extracts of patients suspected of being infected by HPV with a **p53-specific binding DNA fragment; and detecting the amount of wild-type p53 in said cells or cell extract which binds to said DNA fragment, absence of bound p53** indicating infection by strains of HPV which sequester p53.

Drawing Description Text - DRTX (2):

FIGS. 1A and 1B. Screening for **fragments bound by p53** using an immunoprecipitation assay. Panel 1 contains the hFosAva2 clone; panel 2, 772 C.sub.BE ; panel 3, Lambda 5R; panel 4, a pool of clones with inserts of randomly cloned human genomic sequences. 772 C.sub.BE and Lambda 5R contain

Hinfl **fragments (259 and 190 bp, respectively) which bound p53** relatively strongly (arrowheads). "C"--control lane, containing 2% of the labelled DNA used in the binding reactions. "B"--bound DNA recovered from the immunoprecipitate. FIG. 1B. Tests for dependence on p53 and specific antibody. Cell lysates were produced by infection with vaccinia virus that did (+) or did not (-) contain an insert of wild-type p53 cDNA. Immunoprecipitation was performed with anti-p53 monoclonal antibodies (+) or normal mouse IgG (-).

Drawing Description Text - DRTX (3):

FIGS. 2A and 2B. Relative abilities of wild-type and mutant **p53 to precipitate fragment** A. "C"--control lanes, containing 2% of the labelled DNA used in the binding reaction, "B"--bound DNA recovered from the . immunoprecipitate. FIG. 2A. Increasing quantities of wild-type and mutant 273.sup.his p53, affinity-purified from a baculovirus expression system, were used to precipitate labelled C.sub.BE fragments. FIG. 2B. Lysates from a vaccinia virus system (Vac) producing the wild-type (wt), mutant (175.sup.his), or no **p53 protein (-), were used to immunoprecipitate labelled C.sub.BE fragments**. Equivalent quantities of p53 were present in the wild-type and mutant p53 lysates, as assessed by Western blot. In the "Bac" lane, affinity-purified p53 produced in baculovirus-infected insect cells was used in place of the vaccinia-infected lysates.

Drawing Description Text - DRTX (5):

FIGS. 4A and 4B. Binding of various subfragments of **fragments A and B to p53** from vaccinia-infected cell lysates. FIG. 4A. Subfragments of fragment A (subclone 10d) were assayed by immunoprecipitation for their ability to bind wild-type p53 from vaccinia-infected cell lysates. Binding of at least 2% of the DNA added to the reaction was judged as a positive (+) result; lesser but significant binding was recorded as "+/-". Double Lines (=) denote fragment A sequences single lines (-) denote polylinker sequences of the vector, not originally present in fragment A (FIG. 1). Fragment 5mut1 had a G to T transversion at bp 120; 5mut2 had G to T transversions at bp 120 to 122. FIG. 4B. The fragment A (panels 1-4) and fragment B (panel 5) subfragments illustrated in FIG. 4A are labelled to the left of the bands. The "v" band in panel 4 corresponds to the 2.9 kb vector into which subfragment 6 was cloned. Subfragment 8 (panel 5) contained bp 104-238 of fragment B (see FIG. 3B). Control lanes (C) contained 2% of the labelled fragments used in the binding assays (B).

Drawing Description Text - DRTX (8):

FIG. 6B shows Southern blot analysis of transfected clonal lines. The exogenous **p53 gene was present on a 1.8 kb BamHI fragment**. The endogenous p53 gene gave rise to a 7.8 kb BamHI fragment. Other sized fragments presumably arose by rearrangements.

Drawing Description Text - DRTX (11):

FIG. 8. Isolation of **human genomic sequences which bound to p53**.

Drawing Description Text - DRTX (12):
   FIG. 8A. Experimental strategy used for isolation and analysis of human genomic DNA **fragments which bound to p53**.

Drawing Description Text - DRTX (13):
   FIG. 8B. Immunoprecipitation (IP) assays of cloned fragments. Clones of amplified and selected (AS) DNA were tested for the presence of **p53-binding fragments** by IP. For each clone, the bound DNA is shown in the B lane, adjacent to a control (c) lane containing 2% of the total end-labeled DNA used in the binding assay. In this representative experiment, eight binding fragments were identified, representing six unique genomic fragments. The inserts from the clones in lanes labeled 2, 3, 5, 9, 10, and 11 contained **p53-binding fragments,** while the other lanes contained none. The clones in lanes 2 and 5 each contained two binding fragments.

Drawing Description Text - DRTX (18):
   FIG. 11B. Comparison of the ability of wild-type and mutant p53 to bind to the consensus dimer. In vitro translated p53 proteins were tested for the ability to bind the consensus dimer by IP. Two percent of the total DNA used for binding is shown in lane 1. Lane 7 shows binding to baculovirus-produced **human wild-type p53** protein. Lanes 2 to 6 show binding of in vitro translated wild-type and mutant p53 proteins. The mutant p53 proteins contained changes at codon 143 (val to ala), 175 (arg to his), 248 (arg to trp), and 273 (arg to his).

Drawing Description Text - DRTX (23):
   FIG. 13A. Relative DNA-binding abilities of various length concatemers of a p53-binding sequence (PG.sub.n series), using an immunoprecipitation assay. Clones were cleaved by restriction endonucleases to extricate the concatemers, end-labelled, incubated with purified baculovirus-produced wild-type **human p53,** immunoprecipitated with anti-**p53 and protein A-Sepharose, and bound fragments** recovered and separated on a nondenaturing polyacrylamide gel. C, control lane, containing 2% of the labeled DNA used in the binding reactions. B, bound DNA recovered from the binding reactions.

Detailed Description Text - DETX (2):
   It is a finding of this invention that wild-type **p53 protein binds specific fragments** of human chromosomal DNA. Each of the fragments contains more than one monomer of the double-stranded motif 5'-RRRCWWGYYY-3' (SEQ ID NO:3) separated by 0 to 13 bp. Some of these sequences are found near origins of replication of certain animal viruses and animal cells. See Jelinek et al, Proc. Natl. Acad. Sci. USA, vol. 77, p. 1398-1402 (1980). Four mutant forms of **p53 protein which are commonly found in human** tumors do not have the ability to bind to these sequences. Thus, a function of p53 may be mediated by its ability to bind to specific DNA sequences in the human genome.

Detailed Description Text - DETX (5):

It has been found that **p53 will specifically bind to other sequences in the human** genome with similar sequence motifs. Using a strategy coupling immunoprecipitation to "whole-genome PCR" (Kinzler, et al., Nucleic Acids Research, 17:3645-3653 (1989)), twenty **human DNA fragments that bind to p53** have been identified. Each of the fragments contain a sequence which conforms to a dimer of the double-stranded motif 5'-RRRCWWGYYY-3' (SEQ ID NO:3) separated by 0 to 13 bp. These dimers directly mediate binding, as assessed by DNase I protection and methylation interference assays. The consensus dimers contain a striking symmetry, with four 5'-RRRCW-3' (SEQ ID NO:39) units oriented in alternating directions. A synthetic monomer containing the 10 bp consensus sequence is insufficient for binding, while the combination of two or more monomers bind strongly to wt p53, but negligibly to p53 mutants. Thus, more than one monomer appears to be required for binding. The spacing between monomers may be from 0 to 40 nucleotides, although all natural binding sites isolated have spacings of less than 15 nucleotides. The symmetry of the four half-sites within the consensus dimers suggests that p53 interacts with DNA as a tetrameric protein. The twenty unique clones shown in FIG. 10 allow the identification of adjacent genes which may be regulated by p53 and may mediate its growth-suppressive action.

Detailed Description Text - DETX (8):

Based on the sequence information of the **p53 specific-DNA-binding fragments,** a number of diagnostic and therapeutic methods have been devised. According to one such method, cell lysates are tested for the presence or absence of wild-type p53 by virtue of its specific DNA binding ability. As it is known for various cancers and stages of cancers that one or both of the p53 alleles in tumor tissues can be mutant, testing for the presence or absence of wild-type p53 protein can provide diagnostic and prognostic information regarding a tumor and the patient. The cells to be tested are typically isolated from a tissue suspected of being neoplastic. Preferably the tissues are carefully prepared and isolated so that non-neoplastic tissues are not mixed with the neoplastic tissues, which can confound the analysis. Means for separating neoplastic tissues from non-neoplastic tissues are known in the art and include dissection of paraffin or cryostat sections, as well as use of flow cytometry. A cell lysate can be prepared from the tumor tissue according to any method known in the art. The cell lysate is then incubated with DNA **fragments which are known to bind the wild-type p53** protein, under conditions which are conducive to such DNA/protein interactions. Alternatively, a histological sample can be analyzed by incubation with DNA fragments, as described for cell lysates.

Detailed Description Text - DETX (11):

According to another embodiment of the invention, after incubation of **p53 with specific binding DNA fragments** all components of the cell lysate which do not bind to the DNA fragments are removed. This can be accomplished, among other ways, by employing DNA fragments which are attached to an insoluble polymeric support such as agarose, cellulose and the like. After binding, all non-binding components can be washed away, leaving p53 bound to the DNA/solid support. The p53 can be quantitated by any means known in the art. It can be

determined using an immunological assay, such as an ELISA, RIA or Western blotting.

Detailed Description Text - DETX (12):
    The diagnostic assay of the present invention has applicability not only with regard to cancers which are known to involve mutation of **p53, but also with regard to human** viruses such as human papilloma virus (HPV). HPV protein E6 binds tightly to wild-type but not mutant p53. See Werness et al., Science, 248, 76-69 (1990). This tight binding is likely to block the interaction of p53 with its specific DNA binding sequences. By testing cells or cell extracts suspected of being infected with potentially tumor-inducing or hyperplastia-inducing strains of HPV or possible other viruses, infected cells can be identified, because the E6 protein of the infected cells will have sequestered the wild-type p53, rendering it unable to bind to its specific DNA binding sequences. Such assays may be performed on cell extracts or on histological specimens.

Detailed Description Text - DETX (13):
    According to the present invention a method is also provided of supplying wild-type p53 function to a cell which carries mutant p53 alleles. The wild-type p53 gene or a part of the gene may be introduced into the cell in a vector such that the gene remains extrachromosomal. In such a situation the gene will be expressed by the cell from the extrachromosomal location. If the mutant p53 genes present in the cell are expressed, then the wild-type **p53 gene or gene portion** should be expressed to a higher level than that of the mutant gene. This is because the mutant forms of the protein are thought to oligomerize with wild-type forms of the protein. (Eliyahu et al., Oncogene, vol. 3, p. 313, 1988.) If a gene **portion is introduced and expressed in a cell carrying a mutant p53 allele, the gene portion should encode a part of the p53** protein which is required for non-neoplastic growth of the cell. More preferred is the situation where the wild-type p53 gene or a part of it is introduced into the mutant cell in such a way that it recombines with the endogenous mutant p53 gene present in the cell. Such recombination would require a double recombination event which would result in the correction of the p53 gene mutation. Vectors for introduction of genes both for recombination and for extrachromosomal maintenance are known in the art and any suitable vector may be used.

Detailed Description Text - DETX (15):
    According to the present invention compounds which have p53 activity are those which specifically complex with a p53-specific DNA binding site. Wild-type **p53 is one such compound, but portions of p53** which retain the ability to bind to p53-specific binding sites may also be used. Oligonucleotides and oligonucleotide containing nucleotide analogs are also contemplated among those compounds which are able to complex with a p53-specific DNA binding site. Although applicants do not wish to be bound by any particular theory, it is believed that oligonucleotides bind double-stranded DNA to form triplexes. Such triplexes have been shown to block transcription of certain genes, as well as protect the DNA binding sites from the action of enzymes such as DNA methylases. Although originally such

oligonucleotides were thought to require only or predominantly pyrimidenes (cytosine and thymine), purines have also successfully been incorporated into triplex forming oligonucleotides. Particular oligonucleotides which may be used include: nucleotides 140-162 of SEQ ID NO:2, nucleotides 128-158 of SEQ ID NO:1, nucleotides 114-123 of SEQ ID NO: 1, or portions thereof having at least ten nucleotides.

Detailed Description Text - DETX (19):

Double-stranded DNA **fragments which comprise a p53**-specific DNA binding site and are attached to an insoluble polymeric support are also contemplated by this invention. The support may be agarose, cellulose, polycarbonate, polystyrene and the like. Such supported fragments may be used in screens to identify compounds which bind to p53-specific DNA binding sites. Similarly, such supported fragments may be used to perform diagnostic tests on cell lysates from suspected tumor tissues. They may also be used in assays used to screen potential chemotherapeutic agents, as discussed infra.

Detailed Description Text - DETX (20):

Although any method can be employed which utilizes the p53-specific DNA binding sites of the present invention, two particular methods are disclosed for screening for additional compounds that bind to p53-specific DNA binding sites. According to one method a test compound is incubated with a supported DNA fragment, as described above. The amount of test compound which binds to the supported DNA fragment is determined. This determination can be performed according to any means which is convenient. For example, the amount of a compound which can be removed after incubation with the supported fragment can be compared to the amount originally applied. Alternatively, the test compound can be labelled and the amount which binds to the supported fragment can be assayed directly. In order to render this screening method more specific, soluble DNA **fragments which do not contain the p53** DNA binding sequence can be added to the incubation mixture. The soluble **fragments would not have the ability to specifically bind to p53** wild-type protein.

Detailed Description Text - DETX (21):

According to another screening method for compounds to simulate the specific DNA binding activity of **p53, test compounds are incubated with supported DNA fragments** as described above. However, in this method wild-type p53 protein is also added to the incubation mixture. The amount of **p53 protein which binds to the DNA fragment** is measured using methods as described above. The amount of p53 protein bound is compared to the amount which binds in the absence of the test compound. Any diminution of p53 binding which results from the presence of the test compound is presumptively due to the competition of the test compound with **p53 for the specific DNA binding sites of the supported fragments**. Direct binding of the test compound to the binding site fragments can be confirmed using the assay described above.

Detailed Description Text - DETX (24):

Compounds which have p53-specific DNA-binding activity, including wild-type

p53 protein, p lypeptides c rresp nding to p rtions f wild-type p53 protein,
oligonucleotides and oligonucleotide containing nucleotide analogues, as well
as other organic molecules can also be administered to humans and animals as a
pharmaceutical and therapeutic composition. Effective amounts will be
administered to cause neoplastic cells to become less aggressively neoplastic
or even to stop the growth of the neoplastic cells entirely. Generally, such
amounts will be in the range of 10 ng to 10 .mu.g per dose per person or other
animal. The therapeutic compounds can be prepared in any conventional
pharmaceutical excipient, such as physiological saline or other physiologically
compatible aqueous buffer. Typically, the compounds will be administered by
injection, either intravenous or intramuscular. However, other administration
methods as are known in the art and may be used to administer the compounds of
the present invention.

Detailed Description Text - DETX (30):
    In another embodiment of the invention, oligonucleotides can be isolated
which restore to mutant p53 proteins the ability to bind to the consensus
binding sequence or conforming sequences. Mutant p53 protein and random
oligonucleotides are added to a solid support on which **p53-specific-binding DNA
fragments** are immobilized. Oligonucleotides which bind to the solid support
are recovered and analyzed. Those whose binding to the solid support is
dependent on the presence of the mutant p53 protein are presumptively binding
the support by binding to and restoring the conformation of the mutant protein.

Detailed Description Text - DETX (40):
    Each clone was digested with an appropriate restriction endonuclease,
end-labelled with .sup.32 P, and incubated with p53 protein from a lysate of
cells infected with a recombinant vaccinia virus expressing p53 protein.
Labelled DNA **fragments which bound to p53** were then recovered by
immunoprecipitation with monoclonal antibodies against p53. Of the more than
1400 restriction **fragments tested, only two bound reproducibly to p53** under the
experimental conditions used: a 259 basepair Hinfl fragment (fragment A) of
clone 772 C.sub.BE (Panel 2, FIG. 1A), and a 190 basepair Hinfl fragment
(fragment B) of clone Lambda 5R (Panel 3, FIG. 1A); these fragments bound to a
far greater extent than any of the other labelled fragments of larger or
smaller size present in the same assay mixes.

Detailed Description Text - DETX (42):
    This example demonstrates that the immunoprecipitation of **fragment A is
dependent on both p53** protein and anti-p53 antibodies.

Detailed Description Text - DETX (44):
    Lysates from cells infected with wild-type vaccinia virus (devoid of **p53)
were not able to specifically immunoprecipitate fragment** A (FIG. 1B).
Similarly, the detection of the precipitation of **fragment A was dependent on
the presence of anti-p53** antibodies (FIG. 1B). The binding was evident in
lysates prepared from either human HeLa cells or monkey BSC40 cells infected
with vaccinia virus and expressing wild-type p53 (FIG. 1B).

Detailed Description Text - DETX (45):

Affinity-purified baculovirus-produced wild-type p53 protein was substituted for the vaccinia-infected cell lysates in the immunoprecipitation assay and found to bind fragment A strongly (FIG. 2A). This suggested that the binding to **fragment A was an intrinsic property of the p53** polypeptide and not dependent on other factors present in the vaccinia virus-infected cell lysates.

Detailed Description Text - DETX (47):

This example demonstrates that **p53 mutant proteins found in human** tumors fail to bind to fragment A.

Detailed Description Text - DETX (48):

Increasing quantities of wild-type and mutant 273.sup.his p53 protein, affinity purified from a baculovirus expression system, were used to immunoprecipitate labelled fragments from C.sub.BE. See FIG. 2A. The proportion of **fragment A bound to wild-type p53** protein increased in tandem with the amount of p53 added to the assay mixture. (FIG. 2A) In contrast, fragment A did not specifically bind to a mutant form of p53 (273.sup.his) protein even at the highest p53 protein concentration used. The 273.sup.his mutation is the most common **p53 mutant identified in human** tumors. Another p53 mutant (175.sup.his) protein commonly found in human tumors also failed to bind to fragment A (FIG. 1B).

Detailed Description Text - DETX (50):

This example defines the particular sequences within **fragment A that allow it to bind to wild-type p53** protein.

Detailed Description Text - DETX (52):

One primer for each PCR was labelled with .sup.32 P at the 5' end with T4 polynucleotide **kinase** in a 5 .mu.l reaction, and the **kinase** inactivated at 70.degree. C. for 5 min. PCR contained 350 ng of each of the appropriate primers and approximately 50 ng plasmid template in a 50 .mu.l reaction, using 25 cycles and the PCR conditions specified in Baker S J, et al., Cancer. Res., 50:7717 (1990). The products were extracted with phenol and chloroform, ethanol-precipitated, and dissolved in 3 mM Tris, 0.2 mM EDTA prior to binding. Subfragment 1 contained bp 1 to 425 of subclone 10d of **fragment** A (FIG. 3A); subfragments 1a, 1b, 1c, 1d, and 1e were generated by digestion of subfragment 1 with BamHI, MboI, HindIII, HindIII, and BamHI, respectively, from **fragment** 1. Subfragment 2, contained bp 283 to 425. Subfragment 3a was generated by digestion of subfragment 3 (bp 106 to 294) with Hae III. Subfragment 4a was produced from subfragment 4 (gp 1 to 141) by Hind III digestion. Subfragments 5a and 5b were products of the HaeIII digestion of subfragment 5 (bp 87 to 141). "Mutant" subfragments 5mut1 and 5 mut2 were produced using primers P3ml (5'-GAAAGAAAAGGCAAGGCCAGGAAAGT-3') (SEQ ID NO:34) and P3mut2 (5'-GAAAGAAAAGGCAAGGCCATTAAAGT-3') (SEQ ID NO:35) and were identical to subfragment 5 except for the positions underlined in the primers. Subfragment

6 contained bp 106 to 138, and the insert was excised by restriction with HindIII and BamHI to generate 6a or with HindIII and EcoRI to generate 6b. Subfragment 3, including basepairs 106 to 294 (FIG. 4B, panel 2), bound well to **p53** as did subfragment 4, containing basepairs 1 to 141 (FIG. 4B, panel 3). This and similar assays done with additional subfragments (FIGS. 4A and 4B) localized the critical sequences to basepairs 106 to 141. This segment contained three repeats of the sequence TGCCT (SEQ ID NO:36) (FIG. 3A). Digestion of subfragment 3 with HaeIII (cleaving between bp 125-126 and removing two of the TGCCT (SEQ ID NO:36) repeats) greatly reduced this binding (FIG. 4B, subfragment 3A, panel 2), suggesting that a critical sequence lay at or near this restriction site and that a single TGCCT (SEQ ID NO:36) repeat was not sufficient for binding. Additional subfragments were tested (#5, bp 87 to 141, FIGS. 4A and 5B; #6, bp 106 to 138, FIGS. 4A and 4B, panel 4), and it was established that a 33 bp insert containing three TGCCT (SEQ ID NO:36) repeats providing binding capability.

Detailed Description Text - DETX (54):
   This example demonstrates that certain G residues are critical for binding of **p53 to fragment** A.

Detailed Description Text - DETX (58):
   This example defines the region of **fragment B which is important for p53** binding.

Detailed Description Text - DETX (61):
   This example shows that expression of the wild-type **p53 gene in human** colorectal carcinoma cells dramatically inhibits their growth and that a mutant **p53 gene cloned from a human** colorectal carcinoma was incapable of exerting such inhibition.

Detailed Description Text - DETX (69):
   The conclusions made from the above experiments are dependent on the assumption that p53 protein was produced in the transfected cell lines. Clones containing exogenous mutant p53 sequences produced p53 mRNA at a concentration 1.5 to 3.5 times higher than that produced by the endogenous p53 gene (FIGS. 6A and 7A). Immunoblot analysis showed that there was a concomitant small increase in p53 protein expression in the transfectants (1.5- to 3-fold) compared to the untransfected cells. However, this increase was difficult to measure quantitatively, since these cells produced significant amounts of endogenous p53 protein that (unlike endogenous p53 mRNA) could not be distinguished from that produced by the vectors. To confirm that transfected **human cells expressed p53** protein from our constructs, we studied an additional colorectal carcinoma cell line (RKO). RKO cells were obtained through the generosity of M. Brattain. Although RKO cells did not contain a mutation within the susceptible p53 coding sequences, i.e., exons 5-9, they expressed low concentrations of p53 mRNA compared to normal colorectal mucosa or the other lines studied and did not produce detectable amounts of protein.

Detailed Description Text - DETX (70):

Results of colony formation assays in transfected RKO cells were similar to those in SW480 and SW837 cells. Colony formation by wild-type p53 gene transfectants occurred with a tenfold decrease in efficiency compared to the mutant p53 construct (Table 1). Immunocytochemical detection of p53 protein in transfected RKO cells was done as follows: approximately 5.times.10.sup.4 cells were cytocentrifuged onto polylysine-coated slides, fixed for 10 min in formalin, and permeabilized for 5 min in 0.5% Triton X-100. A mouse monoclonal antibody against **human p53** protein (AbI801) in combination with the ABC immunoperoxidase system (Vector Laboratories), was used for immunocytochemical detection of p53 protein (Banks, et al., Eur. J. Biochem. 159, 529 (1986)). Ten to 20 randomly selected microscopic fields were analyzed per slide. These observations are consistent with the greater stability of mutant compared to wild-type p53 protein noted previously (C. A. Finlay et al., Mol. Cell Biol. 8, 531 (1988)). However, transient mRNA expression was also significantly lower in the SN3 transfectants compared to the SCX3 transfectants at 48 and 96 hours, supporting the idea that RKO cells expressing wild-type p53 were at a selective disadvantage compared to those producing mutant p53 products.

Detailed Description Text - DETX (76):

This example demonstrates the identification of human genomic **fragments that can bond to wt p53** protein in vitro.

Detailed Description Text - DETX (78):

Following the outline in FIG. 8A., we tested the inserts of 530 clones for binding to p53. Restriction fragments of the clones were end-labeled and incubated with purified **human wt p53** protein produced in baculovirus-infected cells.

Detailed Description Text - DETX (79):

Whole-genome PCR was performed as previously described, except that only one oligonucleotide (5'-GAGTAGAATTCTAATATCTC-3') (SEQ ID NO:37) was used for amplification (Kinzler, et al. (1989), Nucleic Acids Research, 17:3645-3653, and Kinzler, et al. (1990), Molec. Cell. Biol., 10:634-642). Two hundred ng of "catch"-linked human genomic DNA were incubated with 100 ng of baculovirus-produced **human wt p53** purified as described (Friedman, et al. (1990), Proc. Natl. Acad. Sci. U.S.A., 87:9275-9279), and immunoprecipitated as described below. After 4 rounds of IP and PCR, the AS DNA was cleaved with Eco RI and cloned into either the vector Lambda Zap II or pBluescript II SK+ (Stratagene). Individual clones were picked at random and tested for p53 binding. In panel B, cloned plasmid DNA samples were cleaved with Eco RI and end-labeled by Klenow fill-in. For IP (McKay, et al. (1981), J. Mol. Biol, 145:471-479), ten ng of DNA were incubated with 100 ng of baculovirus-produced **human wt p53** and 100 ng of poly dI-dC at 4.degree. C. for 30 minutes in 100 .mu.l of "DNA-binding buffer" containing 100 mM NaCl, 20 mM Tris, pH 7.0, 10% glycerol, 1% NP40, and 5 mM DTT. DNA **fragments bound to p53** were complexed to antibodies by the addition of 8 .mu.l containing 400 ng each of anti-p53 antibodies pAb421 and pAb1801, both obtained from Oncogene Science, and incubated for 30 minutes at 4.degree. C. The DNA-binding buffer containing 1.5 mg protein A precipitated following the addition of 26 .mu.l of DNA-binding

buffer containing 1.5 mg protein A Sepharose and 10 .mu.g of poly dl-dC and mixing at 4.degree. C. for 30 minutes. After removal of the supernatant, the immunoprecipitate was washed twice with 1 ml of DNA-binding buffer. Bound DNA was purified by treatment with SDS and proteinase K at 48.degree. C. for 30 minutes, extracted with phenol and chloroform, precipitated with ethanol, separated by electrophoresis on a 10% nondenaturing polyacrylamide gel, and autoradiographed.

Detailed Description Text - DETX (80):

Twenty-three of the clones were found to contain **fragments that bound to p53**. Examples of the IP experiments are shown in FIG. 8B. Clone S61 (lanes 11B,C) contains a single genomic **fragment of 202 bp which bound to p53**. Clone N2 contained five **fragments, only one of which (357 bp) bound to p53** (lanes 10B,C). Other examples of **p53-binding fragments** were obtained, and each of these was subcloned for further analysis. In contrast, we found that none of over 1000 clones containing unselected human DNA inserts of similar size bound to p53 using the IP assay. Thus, the whole-genome PCR procedure significant enriched for p53-binding sequences.

Detailed Description Text - DETX (82):

This example demonstrates the localization of **p53 contacts with bound DNA fragments**.

Detailed Description Text - DETX (83):

Localization of the regions bound by p53 was obtained by DP or MI assays using the subcloned DNA fragments as probes. For MI, the **fragments were methylated at G residues and bound to p53** (FIG. 9). methylation of G residues critical for p53 binding resulted in interference with IP. For example, methylation at nucleotides, 217, 22, 227 to 229, and 233 of the 248 bp insert from clone 11B3 completely interfered with the binding of this **fragment to p53** (FIG. 9, footprint 2). When the opposite strand was analyzed, interference was observed at the G residues corresponding to nucleotides 219, 223, 224, 230, 235, and 236 (FIG. 9, footprint 1). For DP, labelled DNA fragments were first subject to IP, then incubated with various amounts of DNase I. For clone N22, p53 binding provided protection against DNase I cleavage at residues 187 to 211 (FIG. 9, footprint 9). MI showed interference by G residues only within the region protected by DNase I (FIG. 9, footprint 10). Other examples of DP and MI mapping are shown in FIG. 9. **p53-binding DNA fragments** were subcloned and labeled on one end, gel-purified and subjected to DP or MI mapping. For MI, 10 ng of DNA were incubated in 200 .mu.l of 50 mM Na-cacodylate, 1 mM EDTA, pH 8.0 and 5 .mu.l of 10% dimethylsulfate/90% ethanol for 5 minutes at 20.degree. C. to methylate G residues. Fifty .mu.l containing 1.5 m Na-Acetate, 1 M .beta.-mercaptoethanol and 60 .mu.g of glycogen were added. The mixture was ethanol-precipitated, washed, and resuspended in 5 .mu.l of 3 mM Tris, 0.2 mM EDTA, pH 7.5, and allowed to bind to wild-type p53 as described in the legend to FIG. 1. After IP and DNA purification, the samples were incubated with 100 .mu.l of 1 M piperidine at 90.degree. C. for 30 minutes. The samples were then dried under vacuum and separated electrophoretically on a 6% polyacrylamide sequencing gel. The control DNA samples were carried through

all incubations except no p53 was added. For these control samples, the protein A Sepharose pellets were treated with SDS and proteinase K without removal of the supernatants (which contained the labeled DNA in the absence of p53).

Detailed Description Text - DETX (84):
For DP assays, end-labeled DNA fragments were immunoprecipitated as described in the legend to FIG. 8. The protein A Sepharose pellets were incubated for two minutes at 25.degree. C. with 200 ng DNase I in 5 mM MgCl.sub.2. After purification of the DNA, as described above, samples were separated by electrophoresis on sequencing gels and loaded as described above for MI. MI was performed on all 18 genomic DNA **fragments which bound to p53**. DP assays were performed on 13 fragments and the regions of protection uniformly coincided with those indicated by the MI assays.

Detailed Description Text - DETX (94):
This example demonstrates that intact **p53 can activate expression in human** cells.

Detailed Description Text - DETX (95):
We first made reporter plasmids (PG.sub.n -CAT series) containing part of the polyomavirus early promoter and the CAT gene located downstream of DNA sequences which could bind to **p53** in vitro (FIG. 8). For the CAT reporters, concatemers of the **p53**-binding region of C.sub.BE were formed by ligation of complementary oligonucleotides, ligated into the EcoRV site of pBluescript II SK+ (Stratagene) to form the PG.sub.n and MG.sub.n series. The BglII-BamHI **fragment** of pPyOICAT (Murakami, et al. (1990) Oncogene, 5:5), containing the polyomavirus early promoter and the CAT gene coding region, was ligated into the BamHI site of the PG.sub.n and MG.sub.n series clones to form the PG.sub.n -CAT and MG.sub.n -CAT series, and the orientation of the inserts characterized by restriction enzyme analysis. The PG.sub.9 -MG.sub.n -CAT and PG.sub.13 -MG.sub.n -CAT series were formed by excising the HindIII-SalI **fragments** of PG.sub.9 -CAT and PG.sub.13 -CAT, blunt-ending, attaching XbaI linkers, and ligating into the XbaI site of the MG.sub.n -CAT series plasmids (where n=1, 5, 10, and 15). For the yeast .beta.-galactosidase reporter plasmids, PG and MG sequences were ligated as SalI-SmaI **fragments** to the SalI and filled-in XhoI sites of pCZ (Buchanan, et al. (1988), Mol. Cell Biol., 8:50806). The construction of the **p53**-wt expression construct has been described (Baker, et al. (1990), Science, 249:912); the mutant expression plasmids were constructed similarly from the previously described cDNA plasmids (Nigro, et al. (1989), Nature, 342:705, and Kern, et al. (1991), Oncogene, 6:131), or in the case of the engineered **phosphorylation** site mutants, by in vitro mutagenesis (Altered Sites, Promega) with verification by sequencing. The construction of the yeast **p53** expression vectors based on pRS314 has been described (Nigro, et al., Mol. Cell Biol. (in press)).

Detailed Description Text - DETX (96):
For the p53 binding sequences, we used a series of concatemers of the

oligonucleotide PG (5'-CCTGCCTGGACTTGCCTGG-3') (SEQ ID NO:40). This contained the binding region of plasmid C.sub.BE, previously shown to bind p53 in vitro. The reporter and an expression vector coding for the intact **human wild-type protein (p53**-wt) (FIG. 12B), were transfected together into the human colorectal cancer cell line HCT 116. This line makes low amounts of apparently wild-type p53 protein.

Claims Text - CLTX (2):

contacting a **p53-specific binding DNA fragment** which conforms to the consensus sequence shown in FIG. 10 (SEQ ID NO:3) with a cell lysate from a tissue of a human, to bind the DNA **fragment to wild-type p53** present in the cell lysate;

Claims Text - CLTX (3):

detecting the presence of wild-type **p53 protein in the cell by detecting binding of the DNA fragment to wild-type p53.**

Claims Text - CLTX (4):

2. The method of claim 1 wherein the **p53-specific binding DNA fragment** comprises nucleotides 103 to 134 as shown in SEQ ID NO:1.

Claims Text - CLTX (5):

3. The method of claim 1 wherein the **p53-specific binding DNA fragment** comprises nucleotides 104 to 123 as shown in SEQ ID NO:1.

Claims Text - CLTX (6):

4. The method of claim 1 wherein the **p53-specific binding DNA fragment** comprises more than one monomer of the sequence 5'-RRRCWWGYYY-3' (SEQ ID NO:3).

Claims Text - CLTX (9):

7. The method of claim 1 wherein the step of detecting binding of the DNA **fragment to wild-type p53** comprises:

Claims Text - CLTX (11):

8. The method of claim 1 wherein the **p53-specific binding DNA fragment** comprises more than two monomers of the sequence TGCCT (SEQ ID NO:36).

Claims Text - CLTX (14):

incubating the section with a detectably-labeled **p53-specific binding DNA fragment** which conforms to the consensus sequence shown in FIG. 10 (SEQ ID NO:3) to bind said DNA **fragment to wild-type p53** present in the tissue sample;

Claims Text - CLTX (17):

10. The method of claim 9 wherein the **p53-specific binding DNA fragment** comprises nucleotides 103 to 134 as shown in SEQ ID NO:1.

Claims Text - CLTX (18):
11. The method of claim 9 wherein the **p53-specific binding DNA fragment** comprises nucleotides 104 to 123 as shown in SEQ ID NO:1.

Claims Text - CLTX (19):
12. The method of claim 9 wherein the **p53-specific binding DNA fragment** comprises more than one monomer of the sequence 5'-RRRCWWGYYY-3' (SEQ ID NO:3).

Claims Text - CLTX (22):
15. The method of claim 9 wherein the **p53-specific binding DNA fragment** comprises more than two monomers of the sequence TGCCT (SEQ ID NO:36).

Claims Text - CLTX (24):
contacting a **p53-specific-binding DNA fragment** which conforms to the consensus sequence shown in FIG. 10 (SEQ ID NO:3) with a test compound to bind the test compound to the DNA fragment;

Claims Text - CLTX (26):
17. The method of claim 16 wherein soluble DNA **fragments are incubated with the test compound and the p53-specific-binding DNA fragment** immobilized on a solid support, said soluble DNA **fragments not having the ability to specifically bind wild-type p53** protein.

Claims Text - CLTX (28):
contacting a **p53-specific-binding DNA fragment** which conforms to the consensus sequence shown in FIG. 10 (SEQ ID NO:3), said fragment immobilized on a solid support with both a test compound and wild-type p53 protein to bind the wild-type **p53 protein to the DNA fragment;**

Claims Text - CLTX (29):
determining the amount of wild-type **p53 protein which is bound to the DNA fragment, inhibition of binding of wild-type p53** protein by the test compound indicating binding of the test compound to the p53-specific DNA binding sequences.

Claims Text - CLTX (31):
contacting a **p53-specific binding DNA fragment** which conforms to the consensus sequence shown in FIG. 10 (SEQ ID NO:3) with a cell lysate from a tissue of a human, to bind the DNA **fragment to wild-type p53** present in the cell lysate, wherein said contacting is done under conditions which favor specific binding over non-specific binding;

Claims Text - CLTX (32):
    detecting the presence of wild-type **p53 protein in the cell by detecting binding of the DNA fragment t   wild-type p53**.

Claims Text - CLTX (36):
    incubating the section with a detectably-labeled **p53-specific binding DNA fragment** which conforms to the consensus sequence shown in FIG. 10 (SEQ ID NO:3) to bind said DNA **fragment to wild-type p53** present in the tissue sample, wherein said incubating is done under conditions which favor specific binding over non-specific binding;

Claims Text - CLTX (41):
    contacting a **p53-specific-binding DNA fragment** which conforms to the consensus sequence shown in FIG. 10 (SEQ ID NO:3) with a test compound to bind the test compound to the DNA fragment, wherein said contacting is done under conditions which favor specific binding over non-specific binding;

Claims Text - CLTX (44):
    contacting a **p53-specific-binding DNA fragment** immobilized on a solid support with both a test compound and wild-type p53 protein to bind the wild-type **p53 protein to the DNA fragment** which conforms to the consensus sequence shown in FIG. 10 (SEQ ID NO:3), wherein said contacting is done under conditions which favor specific binding over non-specific binding;

Claims Text - CLTX (45):
    determining the amount of wild-type **p53 protein which is bound to the DNA fragment, inhibition of binding of wild-type p53** protein by the test compound indicating binding of the test compound to the p53-specific DNA binding sequences.

Other Reference Publication - OREF (5):
    Mercer et al., "Negative Growth Regulation in a Gliblastoma Tumor Cell Line That Conditionally Expresses **Human Wild-Type p53**", Proc. Natl. Acad. Sci. USA 87:6166-6170 (1990).

Other Reference Publication - OREF (10):
    Romano et al., "Identification and Characterization of a **p53 Gene Mutation in a Human** Osteosarcoma Cell Line", Oncogene 4:1483-1488 (1989).

Other Reference Publication - OREF (12):
    Nigro et al., "Mutations in the **p53 Gene Occur in Diverse Human** Tumor Types", Nature 342:705-708 (1989).

US-PAT-NO: 5942235

DOCUMENT-IDENTIFIER: US 5942235 A

TITLE: Recombinant poxvirus compositions and methods of
                inducing immune responses

DATE-ISSUED: August 24, 1999

INVENTOR-INFORMATION:
NAME   CITY   STATE   ZIP CODE COUNTRY
Paoletti; Enzo   Delmar   NY   N/A   N/A

APPL-NO: 08/ 458356

DATE FILED: June 2, 1995

PARENT-CASE:

CROSS REFERENCE TO RELATED APPLICATIONS

   This application is a division of application Ser. No. 08/184,009 , filed
Jan. 19, 1994, which is a continuation-in-part of application Ser. No.
08/007,115, filed Jan. 20, 1993, abandoned, incorporated herein by reference.
Application Ser. No. 08/007,115 is a continuation-in-part of application Ser.
No. 07/847,951, filed Mar. 6, 1992, now abandoned, which in turn is a
continuation-in-part of application Ser. No. 07/713,967, abandoned, filed Jun.
11, 1991 which in turn is a continuation-in-part of application Ser. No.
07/666,056, abandoned, filed Mar. 7, 1991; and, application Ser. No.
08/007,115 is also a continuation-in-part of application Ser. No. 07/805,567,
filed Dec. 16, 1991, now U.S. Pat. No 5,378,457, which in turn is a
continuation-in-part of application Ser. No. 07/638,080, now abandoned, filed
Jan. 7, 1991; and, application Ser. No. 08/007,115 is also a
continuation-in-part of application Ser. No. 07/847,977 filed Mar. 3, 1992,
now abandoned, as a division of application Ser. No. 07/478,179, now
abandoned, filed Feb. 14, 1990 as a continuation-in-part of application Ser.
No. 07/320,471, filed Mar. 8, 1989, now U.S. Pat. No. 5,155,020; all of
which are hereby incorporated herein by reference. Reference is also made to
copending U.S. applications Ser. No. 715,921, filed Jun. 14, 1991, Ser. No.
736,254, filed Jul. 26, 1991, Ser. No. 776,867, filed Oct. 22, 1991, and
Ser. No. 820,077, filed Jan. 13, 1992, all of which are hereby incorporated
herein by reference.

   This application is a continuation-in-part of U.S. application Ser. No.
07/918,278, filed Jul. 22, 1992, now U.S. Pat. No. 5,505,941 which is a
continuation of application Ser. No. 07/537,890, filed Jun. 14, 1990 now U.S.
Pat. No. 5,174,993 issued Dec. 29, 1992 which is a continuation of
application Ser. No. 07/234,390 filed Aug. 23, 1988, now abandoned, which is
a continuation-in-part of application Ser. No. 07/186,054, filed Apr. 25,
1988, now abandoned, which in turn is a continuation-in-part of application
Ser. No. 07/110,335, filed Oct. 20, 1987, now abandoned, which in turn is a

continuation-in-part of application Ser. No. 07/090,711, filed Aug. 28, 1987, now abandoned, and said application Ser. No. 07/537,890 is also a continuation-in-part of application Ser. No. 07/090,209, filed Aug. 27, 1987, now abandoned, which is a division of application Ser. No. 06/622,135, filed Jun. 19, 1984, now U.S. Pat. No. 4,722,848, issued Feb. 2, 1988, which in turn is a continuation-in-part of application Ser. No. 06/446,824, filed Dec. 8, 1982, now U.S. Pat. No. 4,603,112, issued Jul. 29, 1986, which in turn is a continuation-in-part of application Ser. No. 06/334,456, filed Dec. 24, 1981, now U.S. Pat. No. 4,769,330, issued Sep. 6, 1988.

This application is also a continuation-in-part of application Ser. No. 08/306,259, filed Sep. 13, 1994 now U.S. Pat. No. 5,583,028, which is a division of application Ser. No. 08/228,926, filed Apr. 14, 1994, which in turn is a continuation of application Ser. No. 07/881,995, filed May 4, 1992, now abandoned, which in turn is a division of application Ser. No. 07/537,882, filed Jun. 14, 1990, now U.S. Pat. No. 5,110,587, which in turn is a continuation of application Ser. No. 07/090,209, filed Aug. 27, 1987, now abandoned, which in turn is a division of application Ser. No. 06/622,135, filed Jun. 19, 1984, now U.S. Pat. No. 4,722,848, which in turn is a continuation-in-part of application Ser. No. 06/446,824, filed Dec. 8, 1992, now U.S. Pat. No. 4,603,112, which in turn is a continuation-in-part of application Ser. No. 06/334,456, filed Dec. 24, 1981, now U.S. Pat. No. 4,769,330.

US-CL-CURRENT: 424/232.1, 424/199.1 , 424/93.2 , 435/320.1 , 435/456

ABSTRACT:

   Attenuated recombinant viruses containing DNA coding for a cytokine and/or a tumor associated antigen, as well as methods and compositions employing the viruses, are disclosed and claimed. The recombinant viruses can be NYVAC or ALVAC recombinant viruses. The DNA can code for at least one of: human tumor necrosis factor; nuclear phosphoprotein **p53, wildtype or mutant; human** melanoma-associated antigen; IL-2; IFN.gamma.; IL-4; GMCSF; IL-12; B7; erb-B-2 and carcinoembryonic antigen. The recombinant viruses and gene products therefrom are useful for cancer therapy.

15 Claims, 46 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 33

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Abstract Text - ABTX (1):
   Attenuated recombinant viruses containing DNA coding for a cytokine and/or a tumor associated antigen, as well as methods and compositions employing the viruses, are disclosed and claimed. The recombinant viruses can be NYVAC or ALVAC recombinant viruses. The DNA can code for at least one of: human tumor

necrosis factor; nuclear phosphoprotein **p53, wildtype or mutant; human** melanoma-associated antigen; IL-2; IFN.gamma.; IL-4; GMCSF; IL-12; B7; erb-B-2 and carcinoembryonic antigen. The recombinant viruses and gene products therefrom are useful for cancer therapy.

Drawing Description Text - DRTX (41):
   FIG. 39 shows the coding sequence for the **human p53** gene (SEQ ID NO:215).

Detailed Description Text - DETX (374):
   Generation of Poxvirus-based Recombinant Viruses Expressing Wildtype and Mutant Forms of the **Human p53** Gene Product

Detailed Description Text - DETX (375):
   Three plasmids, p53wtXbaISP6/T3, p53-217Xbal, and **p53-238Xbal containing wildtype human p53** gene sequences, and two mutant forms of p53, respectively, were obtained from Dr. Jeffrey Marks (Duke University). The p53-217Xbal contains a p53 gene encoding a p53 product lacking codon 217 while p53-238Xbal encodes a p53 gene product with an cysteine to arginine substitution at amino acid 238. The sequence of the wildtype p53 cDNA and the deduced amino acid sequence was described previously (Lamb and Crawford, 1986; FIG. 3).

Detailed Description Text - DETX (377):
   PCR was also used to generate a 480 bp and 250 bp fragment from p53wtXbaISP6/T3. The 480 bp fragment was derived with oligonucleotides MM003 (SEQ ID NO:92) (5'-GTTTGTATCGTAATGGAGGAGCCGCAGTCAGATC-3') and MM008 (SEQ ID
NO:93)
(5'-CATTACGATACAAACTTAACGGATATCGCGACGCGTTCACACAGGGCAGGTCTTGGC-3')
This
fragment contains the 3' portion of the vaccinia virus H6 promoter sequences and the 5' **portion of the p53** coding sequences through the SgrAI site. The 250 bp fragment was derived by amplification with oligonucleotides MM005 (SEQ ID NO:94) (5'-TACTACCTCGAGCCCGGGATAAAAAACGCGTTCAGTCTGAGTCAGGCCC-3') and MM007 (SEQ
ID NO:95)
(5'-GTGTGAACGCGTCGCGATATCCGTTAAGTTTGTATCGTAATGCAGCTGCGTGGGCGTGA
.
This PCR **fragment contains the 3' end of the p53** coding sequences beginning at the StuI restriction site. The 480 bp and 250 bp PCR fragments were generated such that the 5' end of the MM005/MM007-derived (SEQ ID NO:94/95) fragment overlaps the 3' end of the MM003/MM008-derived (SEQ ID NO:92/93) fragment.

Detailed Description Text - DETX (378):
   The 227 bp, 480 bp, and 250 bp PCR-derived **fragments** were pooled and fused by PCR using oligonucleotides MM006 (SEQ ID NO:96) (5'-ATCATCGGATCCCCCGGGTTCTTTATTCTATAC-3') and MM005 (SEQ ID NO:94). The 783 bp

fused PCR product contains the H6 promoter juxtaposed 5' to the 5' **porti n of the p53** coding sequence (through the SgrAI restriction site) followed by the end of the **p53** coding sequence beginning at the StuI site. Following the end of the **p53** coding sequence, a T.sub.5 NT sequence motif providing early vaccinia transcription **termination** (Yuen and Moss, 1986) and a unique XhoI site were added. It should be noted that the final H6**-p53** PCR fusion product (783 bp) does not contain the **p53** coding sequences between the SgrAI and StuI restriction sites.

Detailed Description Text - DETX (380):
Plasmids containing intact p53 gene (wildtype or mutant forms) juxtaposed 3' to the H6 promoter were generated by first digesting pMM105 with SgrAI and StuI. A 795 bp SgrAI/StuI **fragment was isolated from p53wtXbaISP6/T3 and p53-238Xbal, while a 792 bp fragment was isolated from p53**-217Xbal. These fragments were individually ligated to the SgrAI/StuI digested pMM105 plasmid to yield pMM106, pMM108, and pMM107, respectively.

Detailed Description Text - DETX (383):
ALVAC (CPpp) p53 insertion plasmids were engineered by excising the p53 expression cassettes from pMM106, pMM107, and pMM108 by digestion with BamHI and XhoI and inserting them individually into BamHI/XhoI digested pNVQC5LSP-7. The 1320 bp BamHI/XhoI **fragment containing the H6-p53** expression cassette from pMM106 and pMM108 was inserted into pNVQC5LSP-7 to yield pMM110 and pMM112, respectively, while the 1317 bp BamHI/XhoI fragment derived from pMM107 and inserted into pNVQC5LSP-7 yielded pMM111.

Detailed Description Text - DETX (640):
A **fragment containing the H6 promoted 5' end of the p53** gene fused to the 3' end of the p53 gene was generated by several PCRs as described below.

Detailed Description Text - DETX (643):
PCRIII: Plasmid p11-4 was used as template with oligonucleotides MM084 (SEQ ID NO:212) 5' CAGAAGCTACTACTACTACTACCCACCTGCACAAGCGCC 3' and MM085 (SEQ ID NO:213) 5' AACTACTGTCCCGGGATAAAAATCAGTCTGAGTCAGGCCCCAC 3' to generate a 301 bp fragment. The 301 bp PCR-derived **fragment contains the 3' end of the p53** gene, and the 5' end overlaps the 3' end of the PCRII product. MM084 (SEQ ID NO:212) primes from position 916 of the murine p53 gene toward the 3' end . MM085 (SEQ ID NO:213) primes from position 1173 toward the p53 gene 5' end . The three PCR products were pooled and primed with MM080 and MM085. The resultant 588 bp fragment contains a BamHI site followed by the H6 promoted 5' end of the p53 gene fused to the p53 gene 3' end followed by a SmaI site; the 5' end of the p53 gene ends at the XhoI site at position 37, and the 3' end starts at the SacII site at position 990 (FIG. 38). The 588 bp PCR-derived fragment was digested with BamHI and SmaI generating a 565 bp fragment which was inserted into BamHI/SmaI digested pNC5LSP5 (described below). The resultant plasmid, designated pMM136, was digested with KspI and XhoI to remove a 149 bp fragment,

and the 953 bp KspI/XhoI fragment from p11-4 was inserted. The resultant
plasmid, pMM148, contains the H6 promoted wild-type murine p53 in the ALVAC C5
insertion locus.

Detailed Description Text - DETX (652):
   Insertion of Mutant Forms of **Human P53** into ALVAC and NYVAC

Detailed Description Text - DETX (653):
   Mutant forms of **Human p53** into ALVAC

Detailed Description Text - DETX (654):
   FIG. 18 (Example 15) presented the sequence of the vaccinia H6 promoted
human wild type p53 gene cassette in an ALVAC-based recombinant, vCP207. In
this example, to facilitate description of the mutant forms of the **human p53**
gene being described, FIG. 39 (SEQ ID NO:215) presents only the coding sequence
for the **human wild type p53** gene. The start codon is at position 1 and the
stop codon is at position 1180.

Detailed Description Text - DETX (655):
   Plasmid Cx22A, containing a mutant form of the **human p53** gene, was received
from Arnold Levine (Princeton University, Princeton, N.J.). Relative to the
wild type p53 sequence presented in FIG. 39, the G at nucleotide position 524
is substituted with an A, changing the arg amino acid at codon 175 of the wild
type protein to a his amino acid in Cx22A.

Detailed Description Text - DETX (656):
   Plasmid pMM110 (Example 15, FIG. 18) contains the vaccinia H6 promoted
wildtype **human p53** site. T the ALVAC C5 insertion site. The **human p53** gene
contains two PflmI sites. p53 coding sequences upstream from the first PflmI
site and downstream from the second PflmI site are the same in pMM110 as in
Cx22A. pMM110 was digested with PflmI to remove the 853 central base pairs of
the p53 gene. The 853 bp PflmI fragment from Cx22A containing the base change
at position 524 was inserted. The resultant plasmid, pMM143, contains the H6
promoted mutant p53 gene.

Detailed Description Text - DETX (657):
   Recombination between donor plasmid pMM143 and ALVAC rescuing virus
generated recombinant virus vCP270. vCP270 contains the mutant form of the
human p53 gene under the control of the vaccinia H6 promoter in the C5 locus.

Detailed Description Text - DETX (658):
   Plasmid pR4-2 containing a mutant form of the **human p53** gene was received
from Arnold Levine (Princeton University, Princeton, N.J.). Relative to the
wild type p53 sequence presented in FIG. 39, the G at nucleotide position 818
is substituted by an A, changing the arg codon at amino acid position 273 to a

his codon in pR4-2.

Detailed Description Text - DETX (659):
Plasmid pMM110 (Example 15, FIG. 18) contains the vaccinia H6 promoted **human wildtype p53** gene in the ALVAC C5 insertion site. p53 coding sequences upstream from the first PflmI site and p53 coding sequences downstream from the second PflmI site are the same in pMM110 as in pR4-2. pMM110 was digested with PflmI to remove the 853 central base pairs of the p53 gene. The 853 bp PflmI fragment from pR4-2 containing the base change at nucleotide position 818 was inserted. The resultant plasmid, pMM144, contains the H6 promoted mutant form of the **human p53** gene in the C5 insertion locus.

Detailed Description Text - DETX (660):
Recombination between donor plasmid pMM144 and ALVAC rescuing virus generated recombinant virus vCP269. vCP269 contains the mutant form of the **human p53** gene under the control of the vaccinia H6 promoter in the C5 locus.

Detailed Description Text - DETX (661):
Mutant forms of **Human p53** into NYVAC

Detailed Description Text - DETX (662):
Plasmid Cx22A, described above, contains a mutant form of the **human p53** gene, in which the G at nucleotide position 524 (FIG. 39) is substituted by an A, changing the arg codon at amino acid position 175 to a his codon in Cx22A.

Detailed Description Text - DETX (663):
Plasmid pMM106 (Example 15) contains the vaccinia H6 promoted wild-type **human p53** gene in the NYVAC I4L insertion locus. p53 coding sequences upstream from the first PflmI site and p53 coding sequences downstream from the second PflmI site are the same in pMM106 as in Cx22A. pMM106 was digested with PflmI to remove the 853 central base pairs of the p53 gene. The 853 bp PflmI fragment from Cx22A containing the base change at position 524 was inserted. The resultant plasmid, pMM140, contains the H6 promoted mutant p53 gene.

Detailed Description Text - DETX (664):
Recombination between donor plasmid pMM140 and NYVAC rescuing virus generated recombinant virus vP1234. vP1234 contains the mutant form of the **human p53** gene under the control of the vaccinia H6 promoter in the I4L locus.

Detailed Description Text - DETX (665):
Plasmid pR4-2, described above, contains a mutant form of the **human p53** gene, in which the G at nucleotide position 818 (FIG. 39) is substituted by an A, changing the arg codon at amino acid position 273 to a his codon in pR4-2.

Detailed Description Text - DETX (666):
   pMM106 (Example 15) contains the H6 promoted wild-type **human p53** gene in the
I4L locus. p53 coding sequences upstream from the first PflmI site and p53
coding sequences downstream from the second PflmI site are the same in pMM106
as in pR4-2. pMM106 was digested with PflmI to remove the 853 central base
pairs of the p53 gene. The 853 bp PflmI fragment from pR4-2 containing the
base change at position 818 was inserted. The resultant plasmid, pMM141,
contains the H6 promoted mutant p53 gene.

Detailed Description Text - DETX (667):
   Recombination between donor plasmid pMM141 and NYVAC rescuing virus
generated recombinant virus vP1233. vP1233 contains the mutant form of the
human p53 gene under the control of the vaccinia H6 promoter in the I4L locus.

Detailed Description Text - DETX (668):
   A listing of the wildtype and mutant forms of murine **p53 and the mutant
forms of human p53** present in ALVAC and NYVAC recombinants described in
Examples 31 and 32 is provided in Table 29.

Detailed Description Text - DETX (670):
   ALVAC and NYVAC based recombinants vP1101, vP1096, vP1098, vCP207, vCP193,
vCP191 (all described in Example 15; Table 22, as well as ALVAC and NYVAC based
recombinants vCP270, vCP269, vP1233, vP1234 described in this Example, Table
29), contain wild type or mutant forms of the **human p53** gene. All of these
recombinant virus were assayed for expression of the **human p53** gene using
immunoprecipitation.

Detailed Description Text - DETX (671):
   Recombinant or parental virus were inoculated onto preformed monolayers of
tissue culture cells in the presence of radiolabelled .sup.35 S-methionine and
treated as previously described (Taylor et al., 1990). Immunoprecipitation
reactions were performed using a **human p53** specific monoclonal antibody 1801.
A protein of between 47 and 53 kDa was precipitated from cells infected with
any of the recombinant viruses, vP1101, vP1096, vP1098, vCP207, vCP193, vCP191,
vCP270, vCP269, vP1233, or vP1234, but not from uninfected cells or cells
infected with parental ALVAC or NYVAC virus.

Detailed Description Paragraph Table - DETL (48):
   TABLE 29 _____ Recombinant Virus Parent
Virus Species Gene Insert _____ vCP263 ALVAC
murine w.t. p53 vCP267 ALVAC murine p53 (+3 aa) vCP270 ALVAC **human p53** (aa
175; R to H) vCP269 ALVAC **human p53** (aa 273; R to H) vP1234 NYVAC **human p53**
(aa 175; R to H) vP1233 NYVAC **human p53** (aa 273; R to H)

US-PAT-NO: 5914389

DOCUMENT-IDENTIFIER: US 5914389 A

TITLE: E6 associated protein

DATE-ISSUED: June 22, 1999

INVENTOR-INFORMATION:
NAME	CITY	STATE	ZIP CODE	COUNTRY
Huibregtse; Jon M.	Brighton	MA	N/A	N/A
Scheffner; Martin	Walldorf	N/A	N/A	DE
Howley; Peter M.	Wellesley	MA	N/A	N/A

APPL-NO: 08/ 674030

DATE FILED: July 1, 1996

PARENT-CASE:

This application is a divisional of Ser. No. 08/100,692, filed Jul. 30, 1993 now U.S. Pat. No. 5,532,348, issued Jul. 2, 1996, which is incorporated herein by reference.

US-CL-CURRENT: 530/350, 501/98.4

ABSTRACT:

The present invention provides compositions of isolated and purified E6 Associated Protein and fragments thereof. Also provided are nucleic acid constructs encoding E6 Associated Protein. These compositions may be employed to identify compounds which inhibit binding of high risk HPV E6 to p53. The compositions of the present invention may also be used in methods to detect the presence of high risk HPV in biological samples.

4 Claims, 21 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 12

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Brief Summary Text - BSTX (3):
   Malignant transformation of cells has been linked to expression of proteins encoded by oncogenes (Huang et al., Cell, 39:79-87 (1984)). Cells may also be transformed by suppression of growth- and replication-inhibiting factors. Suppression of these inhibiting factors may allow unrestrained cell replication

and malignant transformation. Lane and Benchimol, Genes and Devel., 4:1-8 (1990). **Human protein p53** is one such inhibiting factor.

Brief Summary Text - BSTX (4):

   Many lines of evidence point to the importance of protein **p53 in human** carcinogenesis. Mutations within the p53 gene are the most frequent genetic aberration thus far associated with human cancer (Vogelstein, Nature, 348:681-682 (1990)) and individuals with germ line p53 mutation have an elevated risk of developing cancer (Malkin et al., Science, 250:1233-1238 (1990); Srivastava et al., Nature, 348:747-749 (1990)). The mutations identified in cancers are generally point mutations which fall within evolutionarily conserved domains and most of these mutated alleles have transforming activity in various cell culture assays (reviewed in Lane and Benchimol, supra and Levine et al., Nature, 351:453-456 (1991)).

Brief Summary Text - BSTX (15):

   Methods for detecting human papillomavirus associated with a high risk of malignancy in a biological sample are also provided. The methods generally comprise contacting the sample with a composition comprising mammalian tumor suppressor protein p53 and purified E6 Associated Protein under conditions conducive to complex formation, detecting formation of complexes between E6 and **p53 therein, and determining the presence of human** papillomavirus infection associated with a high risk of malignancy. The present invention also provides methods for identifying compounds which inhibit binding of E6 to p53. The methods generally comprise contacting compounds of interest with isolated and purified E6-AP to form a mixture, adding the mixture to a composition comprising E6 and p53, but not containing E6 Associated Protein, and detecting formation of complexes of E6 and p53.

Detailed Description Text - DETX (7):

   These E6-AP peptides of the present invention may be employed to inhibit binding of high risk E6 proteins to p53. By "high risk E6 proteins", it is meant E6 proteins produced by papillomaviruses associated with a high risk of malignant degeneration following cellular infection. Such "high risk E6 proteins" include E6 proteins from human papillomaviruses 16 or 18. **Peptides of the present invention may be synthesized which will bind E6, p53,** or both. This may block the E6 mediated degradation of p53. Further, E6-AP proteins having mutations in regions necessary for p53 degradation by ubiquitination may be synthesized to block p53 ubiquitination.

Detailed Description Text - DETX (66):

   E6-AP can stably associate with high risk HPV E6 proteins in the absence of **p53**. The largest segment of E6-AP fused to GST consisted of the C-**terminal** 653 amino acids (amino acids 212-865 of SEQ ID NO:1). This 75 kDa **fragment** of E6-AP contains all of the sequences necessary to direct association of E6 with **p53** and induce degradation of **p53** by ubiquitination. Equal amounts (approximately 0.1 .mu.g) of different GST-E6-AP **fragment** fusion proteins were assayed for the ability to associate with HPV16 or HPV11 E6 proteins by mixing

the GST fusion proteins, immobilized on glutathione-Sepharose, with in vitro-translated .sup.35 S-labeled E6 proteins. Wheat germ extract was used for translation of E6 proteins. The level of binding considered non-specific was that amount of E6 protein that bound to GST lacking E6-AP protein sequences. FIG. 8A illustrates a schematic representation of the regions of E6-AP assayed for E6 association. FIG. 8B illustrates binding of the fusion proteins to labeled E6. The 75 kDa form of the E6-AP and the amino-**terminal portion** (amino acids 213 to 489 of SEQ ID NO:1) of the this region bound specifically to HPV16 E6. The carboxy-**terminal portion** of E6 -AP (amino acids 544 to 865 of SEQ ID NO:1) did not bind to HPV16 E6. Binding assays of additional fusion proteins containing amino acid sequences of the amino-**terminal** region demonstrated that the E6 binding domain is between amino acids 371 and 440 of SEQ ID NO:1 (also referred to as SEQ ID NO:2). This E6-AP **fragment** is encoded by SEQ ID NO:5. These fusion proteins bound approximately 50% of the input .sup.35 S-labeled HPV16 E6. None of the E6-AP fusion proteins bound HPV11 E6 above the background level.

Detailed Description Text - DETX (75):

   p53 ubiquitination assays were performed by combining 2 .mu.l , of .sup.35 S-labeled wheat germ extract-translated **human wild-type p53** with 10 .mu.l of either a mock wheat germ extract translation reaction mixture or a wheat germ extract translation mixture programmed with HPV16 E6 mRNA and with 10 .mu.l of the DEAE fraction from either uninfected S19 cells or baculovirus-infected S19 cells. Additionally, each reaction mixture contained 24 .mu.l of T.sub.25 N.sub.50, 2 .mu.l of 2 mg/ml ubiquitin (Sigma), and 2 .mu.l of 40 mM ATP-.gamma.-S (total volume, 50 .mu.l). The mixtures were incubated at room temperature for 4 hours and then analyzed by SDS-PAGE and fluorography.

Other Reference Publication - OREF (16):

   Huibregtse et al., "A Cellular Protein Mediates Association of **p53 with the E6 Oncoprotein of Human** Papillomavirus Types 16 or 18", EMBO J., 10:4129-4135 (1991).

Other Reference Publication - OREF (18):

   Scheffner et al., "The state of the **p53 and retinoblastoma genes in human** cervical carcinoma cell lines", Proc. Natl. Acad. Sci. USA, 88:5523-5527 (1991).

Other Reference Publication - OREF (23):

   Oliner et al., "Amplification of a Gene Encoding a **p53-associated Protein in Human** Sarcomas", Nature, 358:80-83 (Jul. 2, 1992).

Other Reference Publication - OREF (27):

   Huibregtse, et al., "Cloning and Expression of the cDNA for E6-AP, a Protein That Mediates the Interaction of the **Human Papillomavirus E6 Oncoprotein with p53,**" Mol. Cell. Biol., 13(2):775-784 (Feb., 1993).

US-PAT-NO: 5858976

DOCUMENT-IDENTIFIER: US 5858976 A
See image for Certificate of Correction

TITLE: Methods for inhibiting interaction of **human MDM2 and p53**

DATE-ISSUED: January 12, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Burrell; Marilee	Cambridge	MA	N/A	N/A
Hill; David E.	Arlington	MA	N/A	N/A
Kinzler; Kenneth W.	Baltimore	MD	N/A	N/A
Vogelstein; Bert	Baltimore	MD	N/A	N/A

APPL-NO: 08/ 801718

DATE FILED: February 14, 1997

PARENT-CASE:

   This application is a continuation of application Ser. No. 08/390,515,
filed on Feb. 17, 1995 now U.S. Pat. No. 5,765,455, which is a division of
U.S. Pat. No. 5,420,263 (08/044,619), filed Apr. 7, 1993, which is a
continuation-in-part of U.S. Pat. No. 5,411,860 (07/903,103) filed Jun. 23,
1992, which is a continuation-in-part of Ser. No. 07/867,840 (abandoned) filed
Apr. 7, 1992.

US-CL-CURRENT: 514/12, 530/324 , 530/350

ABSTRACT:

   A human gene has been discovered which is genetically altered in human tumor
cells. The genetic alteration is gene amplification and leads to a
corresponding increase in gene products. Detecting that the gene, designated
hMDM2, has become amplified or detecting increased expression of gene products
is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and
allows the cell to escape from p53-regulated growth.

5 Claims, 24 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 18

---------- KWIC ---------

Abstract Text - ABTX (1):
    A human gene has been discovered which is genetically altered in human tumor
cells. The genetic alteration is gene amplification and leads to a
corresponding increase in gene products. Detecting that the gene, designated
hMDM2, has become amplified or detecting increased expression of gene products
is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and
allows the cell to escape from p53-regulated growth.

TITLE - TI (1):
    Methods for inhibiting interaction of **human MDM2 and p53**

Brief Summary Text - BSTX (6):
    While there exists an enormous body of evidence linking **p53 gene mutations
to human** tumorigenesis (Hollstein et al., 1991, Science 253:49-53) little is
known about cellular regulators and mediators of p53 function.

Brief Summary Text - BSTX (7):
    Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that
p53 cDNA clones, containing a point mutation at amino acid residue 143, 175,
273 or 281, cooperated with the activated ras oncogene to transform primary rat
embryo fibroblasts in culture. These mutant p53 genes are representative of
the majority of mutations found in human cancer. Hollstein et al., 1991,
Science 253:49-53. The transformed fibroblasts were found to produce elevated
levels of **human p53** protein having extended half-lives (1.5 to 7 hours) as
compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Brief Summary Text - BSTX (8):
    Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric
protein complex with the cellular heat shock protein hsc70. While residue 273
or 281 mutants do not detectably bind hsc70, and are poorer at producing
transformed foci than the 175 mutant, complex formation between mutant p53 and
hsc70 is not required for p53-mediated transformation. Complex formation does,
however, appear to facilitate this function. All cell lines transformed with
the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the
wild-type **human p53** gene does not possess transforming activity in cooperation
with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

Brief Summary Text - BSTX (9):
    Hinds et al., supra also expressed **human p53** protein in transformed rat
cells. When the expressed **human p53** was immunoprecipitated with two p53
specific antibodies directed against distinct epitopes of p53, an unidentified
M.sub.r 90,000 protein was coimmunoprecipitated. This suggested that the rat
M.sub.r 90,000 protein is in a complex with the **human p53** protein in the
transformed rat cell line.

Brief Summary Text - BSTX (19):

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of **human MDM2 t   human p53**.

Brief Summary Text - BSTX (22):
   Still another object of the invention is to provide polypeptides which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (23):
   It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to **human p53** in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

Drawing Description Text - DRTX (8):
   FIG. 6 shows the determination of MDM2 and p53 domains of interaction. FIG. 5A and FIG. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS3 14SN (p53 expression vector) and pJK103 (lexA-responsive .beta.-galactosidase reporter). Yeast clones expressing .beta.-galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined. .beta.-galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. FIG. 6C. Random **fragments of p53** were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Detailed Description Text - DETX (12):
   It has been found that amino acid residues 13-41 of **p53** (See SEQ ID NO: 1) are necessary for the interaction of MDM-2 and **p53**. However, additional residues on either the amino or carboxy **terminal** side of the **peptide** appear also to be required. Nine to 13 additional **p53** residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from **p53**-regulated growth control in sarcomas, the use of **p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53**-regulated growth control.

Detailed Description Text - DETX (13):
   Suitable **p53-derived peptides** for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

Detailed Description Text - DETX (14):

DNA encoding the MDM2-binding, **p53-derived peptide,** or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus, or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, inter alia encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of **peptide, the goal is to alleviate the sequestration of p53** by MDM2.

Detailed Description Text - DETX (17):

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to **human p53**. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

Detailed Description Text - DETX (26):

To determine whether the hMDM2 protein could bind to **human p53** protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in FIG. 1 (SEQ ID NO:2) from nucleotide 312 to 2176. A 42 bp black beatle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Detailed Description Text - DETX (32):

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (FIG. 2, lanes 2 and 3). However, when in vitro translated **human p53** was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association in vitro (FIG. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (FIG. 2, lanes 8 and 9). When an in vitro translated mutant form of p53 (175.sup.his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

Detailed Description Text - DETX (58):
   This assay was then applied to mapping the interaction domains of each
protein. Full length cDNA **fragments enc ding MDM2 or p53** were randomly sheared
by sonication, amplified by polymerase chain reaction, size fractionated,
cloned into the appropriate fusion vectors and transfected into yeast along
with the reporter and the full length version of the other protein.

Detailed Description Text - DETX (59):
   METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested
with XhoI and BamHI to excise the entire insert. After agarose gel
purification, the insert was sheared into random fragments by sonication,
polished with the Klenow fragment of DNA polymerase I, ligated to catch
linkers, and amplified by the polymerase chain reaction as described (Kinzler,
K. W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were
fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000
pb, cloned into lexA(1-202)+PL (Ruden, D. M., et al., Nature 350:250-252
(1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least
10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was
transfected into a strain of pEGY48 containing pRS314N (p53 expression vector)
and pJK103 (lexA-responsive .beta.-galactosidase reporter). Approximately
5,000 yeast clones were plated on selective medium containing 2% dextrose, and
were replica-plated onto glalctose- and X-gal-containing selective medium.
Blue colonies (17) appeared only on the plates containing the larger fragments
of MDM2. The 17 isolated colonies were tested for blue color in this assay
both in the presence and in the absence of galactose (p53 induction); all
tested positive in the presence of galactose but only 2 of the 17 tested
positive in its absence. MDM2-containing plasmid DNA extracted from the 17
yeast clones was selectively transferred to bacterial strain KC8 and sequenced
from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent
clones are diagrammed in FIG. 6A. The MDM2 sequences of the remaining 15
p53-dependent clones coded for peptides ranging from 135 to 265 a.a. in length
and began exclusively at the initiator methionine. Three of the MDM2 sequences
obtained are shown at the top of FIG. 6B. The lower 6 sequences were
genetically engineered (using the polymerase chain reaction and appropriate
primers) into lexA(1-202)+PL and subsequently tested to further narrow the
binding region.

Detailed Description Text - DETX (60):
   Fragments of p53 were also cloned into pJG4-5, producing a fusion protein
C-**terminal** to the B42 acidic activation domain and incorporating an epitope of
hemagglutinin. The clones were transfected into a strain of pEGY48 already
containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The
top three **p53** sequences shown in FIG. 6C. were derived from yeast obtained by
colony screening, whereas the lower three were genetically engineered to
contain the indicated **fragments**.

Detailed Description Text - DETX (61):
   The resultant yeast colonies were examined for .beta.-galactosidase activity
in situ. Of approximately 5000 clones containing MDM2 **fragments** fused to the
lexA DNA binding domain, 17 were found to score positively in this assay. The

clones could be placed into two classes. The first class (two clones) expressed low levels of .beta.-galactosidase (about 5-fold less than the other fifteen clones) and .beta.-galactosidase. expression was independent of **p53** expression (FIG. 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable .beta.-galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino **terminal** region of MDM2 (FIG. 6B). The .beta.-galactosidase activity of these clones was dependent on **p53** co-expression. To narrow down the region. of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length **p53** contained MDM2 codons 1 to 118 (FIG. 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated **fragments** of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

Detailed Description Text - DETX (62):

In a converse set of experiments, yeast clones containing **fragments of p53** fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (FIG. 2C SEQ ID NO:1)The minimal overlap between these three **fragments** contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the **fragments** required 9-12 amino acids on either side of codons 13-41 for activity (FIG. 6C). To further test the idea that the amino **terminal** region of **p53** was required for MDM2 binding, we generated an additional yeast strain expressing the lex-A-DNA binding domain fused to **p53** codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-**terminus of p53** were required for the interaction.

Detailed Description Text - DETX (63):

Sequence analysis showed that all **p53 and MDM2 fragments** noted in FIG. 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in FIG. 6 expressed the relevant proteins at similar levels, as shown by Western blotting (FIG. 7).

Claims Text - CLTX (2):

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting of a **portion of p53, said portion c mprising amin acids 13-41 of p53** as shown in SEO ID NO: 1, said polypeptide being capable of binding to human MDM2 as shown in SEO ID NO: 3.

Claims Text - CLTX (6):
   administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting of a **portion of p53, said portion comprising amino acids 13-41 of p53** as shown in SEQ ID NO: 1, wherein said polypeptide lacks the homo-oligomerization domain of **p53, and said polypeptide is capable of binding to human** MDM2 as shown in SEQ ID NO:3.

Claims Text - CLTX (8):
   administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting of a **portion of p53, said portion comprising amino acids 13-41 of p53** as shown in SEQ ID NO: 1, said polypeptide being capable of binding to human MDM2 as shown in SEQ ID NO:3, wherein said polypeptide lacks amino acids 138-393 of **p53, and said polypeptide is capable of binding to human** MDM2.

Other Reference Publication - OREF (9):
   Hinds, et al., "Mutant **p53 DNA Clones From Human** Colon Carcinomas Cooperate With Ras in Transforming Primary Rat Cells: A Comparison of the Hot Spot Mutant Phenotypes", Cell Growth & Differentiation, 1:561-580 (1990).

Other Reference Publication - OREF (12):
   Oliner, et al., "Amplification of a Gene Encoding a **p53-Associated Protein in Human** Sarcomas", Nature, 358:80-83 (1992).

Other Reference Publication - OREF (14):
   Leach, et al., "**p53 Mutation and MDMS Amplification in Human** Soft Tissue Sarcomas", Cancer Research 53:2231-2234 (1993).

US-PAT-NO: 5843684

DOCUMENT-IDENTIFIER: US 5843684 A
See image for Certificate of Correction

TITLE: Method for detecting pre-cancerous or cancerous cells
                using P90 antibodies or probes

DATE-ISSUED: December 1, 1998

INVENTOR-INFORMATION:
NAME   CITY   STATE   ZIP CODE COUNTRY
Levine; Arnold J.   Princeton   NJ   N/A   N/A
Finlay; Cathy A.   Chapel Hill   NC   N/A   N/A
Cordon-Cardo; Carlos New York   NY   N/A   N/A

APPL-NO: 08/ 362590

DATE FILED: March 31, 1995

PARENT-CASE:

   This application is a continuation-in-part of PCT Application No.
PCT/US93/06163, filed Jun. 28, 1993, which is a continuation-in-part of U.S.
Ser. No. 08/018,649, filed Feb. 17, 1993, now abandoned, which is a
continuation-in-part of U.S. Ser. No. 07/904,766, filed Jun. 26, 1992, now
abandoned, which in turn is a continuation-in-part of U.S. Ser. No.
07/730,185, filed Jul. 12, 1991, now abandoned, which in turn is a
continuation-in-part of U.S. Ser. No. 07/543,963 filed Jun. 27, 1990, now
abandoned, all of which are incorporated herein by reference.

PCT-DATA:
 APPL-NO: PCT/US93/06163
 DATE-FILED: June 28, 1993
 PUB-NO: WO94/00603
 PUB-DATE: Jan 6, 1994
 371-DATE: Mar 31, 1995
 102(E)-DATE:Mar 31, 1995

US-CL-CURRENT: 435/7.23, 435/326 , 435/6 , 436/64 , 436/813 , 530/387.7
            , 530/388.8 , 530/388.85

ABSTRACT:

   The invention provides a method of diagnosing cancer by determining the
expression level or gene amplification of p53 and dm2, whereby an elevated
level of either p53 or dm2 or both p53 and dm2 indicates a cancer diagnosis.
Furthermore, the invention provides a method of predicting the progress of
cancer by determining the expression level or gene amplification of p53 and
dm2, whereby an elevated level of either p53 or dm2 or both p53 and dm2

indicated a poor prognosis.

34 Claims, 4 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 4

---------- KWIC ---------

Brief Summary Text - BSTX (12):
   The human homolog of the mdm2 gene, called the hdm2 gene or MDM2 or MDM2,
has been cloned and mapped to the long arm of chromosome 12 (12q13-14) (Oliner
et al. 1992. Amplification of a gene encoding a **p53-associated protein in
human** sarcomas. Nature 358:80-83). This region contains two genes, SAS and
GLI, previously found to be amplified in osteo- and soft tissue sarcomas. The
SAS gene codes for a protein of unidentified function. It was isolated from a
malignant fibrous histiocytoma (MFH), and was shown to be amplified in MFH and
liposarcomas (Turc-Carel, C. et al. (1986) Cancer Genet Cytogenet 23, 291-299;
Meltzer, P. S. et al. (1991) Cell Growth Diff 2, 495-501). The GLI gene codes
for a DNA-binding zinc finger protein. Even though it was originally isolated
from a glioblastoma, it has also been reported to be amplified in a
rhabdomyosarcoma and an osteosarcoma (Kinzler, K. et al. (1984) Science 236,
70-73).

Detailed Description Text - DETX (36):
   Briefly, polyclonal antibodies may be produced by injecting a host mammal,
such as a rabbit, mouse, rat, or goat, with the **p53 protein or a fragment**
thereof capable of producing antibodies that distinguish between mutant p53 and
wild-type p53. The peptide or peptide fragment injected may contain the
wild-type sequence or the mutant sequence. Sera from the mammal are extracted
and screened to obtain polyclonal antibodies that are specific to the peptide
or peptide fragment. The same method may be applied to dm2 proteins.

Detailed Description Text - DETX (40):
   Suitable antibodies for the co-immunoprecipitation of p53 and dm2 include
PAb421 and Ab2. PAb421 recognizes the carboxy-terminus of **p53 from various
species, including human, mouse and rat p53,** and is described by Harlow et al.
in the Journal of Virology 39, 861-869 (1981). Ab2 is specific for the
amino-terminus of **human p53,** and is available from Oncogene Science, Inc. of
Manhassett, N.Y. The dm2 protein does not immunoprecipitate when REF cells
that do not express p53 are treated in the same way with the same antibodies.

Detailed Description Text - DETX (79):
   A panel of mouse monoclonal antibodies to the p90 gene encoded gene product
were used for the present study. Antibody 4B2 detects an epitope located in
the amino-**terminal** region. Antibodies 2A9 and 2A10 identify two distinct
epitopes in the central **portion** of p90. Antibody 4B11 recognizes a sequence

located in the carboxy-**terminal** region of p90. Three mouse monoclonal antibodies detecting different epitopes on **p53** proteins were used for the present study. Anti-**p53** antibody PAb1801 (Ab-2, Oncogene Science, Manhasset, N.Y.) recognizes an epitope located between amino acids (aa) 32 to 79 of both wild-type and mutant **human p53** proteins (Banks, L. et al. (1986) Eur J Biochem 159, 529-534). Antibody PAb240 (Ab-3, Oncogene Science) recognizes a conformational epitope located between aa 156 to 335 characteristic of certain mutant **p53** products (Gannon, J. V. et al. (1990) EMBO J 9, 1595-1602). Antibody PAb1620 (Ab-5, Oncogene Science) reacts specifically with wild type **p53** (Ball, R. K. et al. (1984) EMBO J 3, 1485-1491). MlgS-Kp I, a mouse monoclonal antibody of the same subclass as the anti-p90 and anti-**p53** antibodies, was used as a negative control at similar working dilutions.

Detailed Description Text - DETX (85):

These studies were performed according to a slight modification of the method reported by Orita et al (Orita, M. et al. (1989) Genomics 5, 874-879). Amplifications were performed using 100 ng of genomic DNA extracted from the samples described above. The primers used were obtained from intronic sequences flanking exons 5 through 9 of the **human p53** gene, sequences being previously published (Moll, U. M. et al. (1992) Proc Natl Acad Sci USA 89, 7262-7266). DNA was amplified following 30 cycles of PCR (30s at 94.degree. C., 30s at 58.degree. C. for exons 8 and 9 and 63.degree. C. for exons 5, 6 and 7, and finally 60s for all samples at 72.degree. C.) using a Thermal Cycler (Perkin Elmer Cetus). Amplified samples were then denatured and loaded onto a non-denaturing acrylamide gel containing 10% glycerol and run at room temperature for 12 -16 hours at 10-12 watts. Gels were dried at 80.degree. C. under vacuum and exposed to X-ray film at -70.degree. C. for 4-16 hours.

US-PAT-NO: 5833975

DOCUMENT-IDENTIFIER: US 5833975 A

TITLE: Canarypox virus expressing cytokine and/or
                tumor-associated antigen DNA sequence

DATE-ISSUED: November 10, 1998

INVENTOR-INFORMATION:
NAME	CITY	STATE	ZIP CODE	COUNTRY
Paoletti; Enzo	Delmar	NY	N/A	N/A
Tartaglia; James	Schenectady	NY	N/A	N/A
Cox; William I.	Troy	NY	N/A	N/A

APPL-NO: 08/ 184009

DATE FILED: January 19, 1994

PARENT-CASE:

   CROSS REFERENCE TO RELATED APPLICATIONS

   This application is a continuation-in-part of application Ser. No.
08/007,115 now abandoned, filed Jan. 21, 1993, incorporated herein by
reference. Application Ser. No. 08/007,115, now abandoned, is a
continuation-in-part of application Ser. No. 07/847,951, now abandoned, filed
Mar. 6, 1992, which in turn is a continuation-in-part of application Ser. No.
07/713,967, now abandoned, filed Jun. 11, 1991 which in turn is a
continuation-in-part of application Ser. No. 07/666,056, now abandoned, filed
Mar. 7, 1991; and, application Ser. No. 08/007,115, now abandoned, is also a
continuation-in-part of application Ser. No. 07/805,567, now abandoned, filed
Dec. 16, 1991, which in turn is a continuation-in-part of application Ser.
No. 07/638,080, now abandoned, filed Jan. 7, 1991; and, application Ser. No.
08/007,115, now abandoned is also a continuation-in-part of application Ser.
No. 07/847,977, now abandoned, filed Mar. 3, 1992 as a division of application
Ser. No. 07/478,179, now abandoned, filed Feb. 14, 1990 as a
continuation-in-part of application Ser. No. 07/320,471, now U.S. Pat. No.
5,155,020 filed Mar. 8, 1989; all of which are hereby incorporated herein by
reference. Reference is also made to copending U.S. applications Ser. Nos.
715,921, filed Jun. 14, 1991, 736,254, filed Jul. 26, 1991, 776,867, filed
Oct. 22, 1991, and 820,077, filed Jan. 13, 1992, all of which are hereby
incorporated herein by reference.

US-CL-CURRENT: 424/93.2, 435/320.1 , 435/456 , 435/69.3 , 435/69.5
          , 435/69.51 , 435/69.52

ABSTRACT:

   Attenuated vaccinia or canarypox recombinant viruses containing DNA coding

for a cytokine and/or a tumor associated antigen, as well as methods and compositions employing the viruses, are disclosed and claimed. The recombinant viruses can be NYVAC or ALVAC recombinant viruses. The DNA can code for at least one of: human tumor necrosis factor; nuclear phosphoprotein **p53, wildtype r mutant; human** melanoma-associated antigen; IL-2; IFN.gamma.; IL-4; GMCSF; IL-12; B7; erb-B-2 and carcinoembryonic antigen. The recombinant viruses and gene products therefrom are useful for cancer therapy.

5 Claims, 46 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 33

---------- KWIC ---------

Abstract Text - ABTX (1):
    Attenuated vaccinia or canarypox recombinant viruses containing DNA coding for a cytokine and/or a tumor associated antigen, as well as methods and compositions employing the viruses, are disclosed and claimed. The recombinant viruses can be NYVAC or ALVAC recombinant viruses. The DNA can code for at least one of: human tumor necrosis factor; nuclear phosphoprotein **p53, wildtype or mutant; human** melanoma-associated antigen; IL-2; IFN.gamma.; IL-4; GMCSF; IL-12; B7; erb-B-2 and carcinoembryonic antigen. The recombinant viruses and gene products therefrom are useful for cancer therapy.

Drawing Description Text - DRTX (41):
    FIG. 39 shows the coding sequence for the **human p53** gene (SEQ ID NO:215).

Detailed Description Text - DETX (383):
    Generation of Poxvirus-based Recombinant Viruses Expressing Wildtype and Mutant Forms of the **Human p53** Gene Product

Detailed Description Text - DETX (384):
    Three plasmids, p53wtXbaISP6/T3, p53-217Xbal, and **p53-238Xbal containing wildtype human p53** gene sequences, and two mutant forms of p53, respectively, were obtained from Dr. Jeffrey Marks (Duke University). The p53-217Xbal contains a p53 gene encoding a p53 product lacking codon 217 while p53-238Xbal encodes a p53 gene product with an cysteine to arginine substitution at amino acid 238. The sequence of the wildtype p53 cDNA and the deduced amino acid sequence was described previously (Lamb and Crawford, 1986; FIG. 3).

Detailed Description Text - DETX (386):
    PCR was also used to generate a 480 bp and 250 bp fragment from p53wtXbaISP6/T3. The 480 bp fragment was derived with oligonucleotides MM003 (SEQ ID NO:92) (5'-GTTTGTATCGTAATGGAGGAGCCGCAGTCAGATC-3') and MM008 (SEQ ID

NO:93)
(5'-CATTACGATACAAACTTAACGGATATCGCGACGCGTTCACACAGGGCAGGTCTTGGC-3')
This
fragment contains the 3' portion of the vaccinia virus H6 promoter sequences
and the 5' **portion of the p53** coding sequences through the SgrAI site. The 250
bp fragment was derived by amplification with oligonucleotides MM005 (SEQ ID
NO:94) (5'-TACTACCTCGAGCCCGGGATAAAAAACGCGTTCAGTCTGAGTCAGGCCC-3') and
MM007 (SEQ
ID NO:95)
(5'-GTGTGAACGCGTCGCGATATCCGTTAAGTTTGTATCGTAATGCAGCTGCGTGGGCGTGA
C-3'). This PCR **fragment contains the 3' end of the p53** coding sequences
beginning at the StuI restriction site. The 480 bp and 250 bp PCR fragments
were generated such that the 5' end of the MM005/MM007-derived (SEQ ID
NO:94/95) fragment overlaps the 3' end of the MM003/MM008-derived (SEQ ID
NO:92/93) fragment.

Detailed Description Text - DETX (387):
   The 227 bp, 480 bp, and 250 bp PCR-derived **fragments** were pooled and fused
by PCR using oligonucleotides MM006 (SEQ ID NO:96)
(5'-ATCATCGGATCCCCCGGGTTCTTTATTCTATAC-3') and MM005 (SEQ ID NO:94). The
783 bp
fused PCR product contains the H6 promoter juxtaposed 5' to the 5' **portion of
the p53** coding sequence (through the SgrAI restriction site) followed by the
end of the **p53** coding sequence beginning at the StuI site. Following the end
of the **p53** coding sequence, a T.sub.5 NT sequence motif providing early
vaccinia transcription **termination** (Yuen and Moss, 1986) and a unique XhoI site
were added. It should be noted that the final H6-**p53** PCR fusion product (783
bp) does not contain the **p53** coding sequences between the SgrAI and StuI
restriction sites.

Detailed Description Text - DETX (389):
   Plasmids containing intact p53 gene (wildtype or mutant forms) juxtaposed 3'
to the H6 promoter were generated by first digesting pMM105 with SgrAI and
StuI. A 795 bp SgrAI/StuI **fragment was isolated from p53wtXbaISP6/T3 and
p53-238Xbal, while a 792 bp fragment was isolated from p53**-217Xbal. These
fragments were individually ligated to the SgrAI/StuI digested pMM105 plasmid
to yield pMM106, pMM108, and pMM107, respectively.

Detailed Description Text - DETX (392):
   ALVAC (CPpp) p53 insertion plasmids were engineered by excising the p53
expression cassettes from pMM106, pMM107, and pMM108 by digestion with BamHI
and XhoI and inserting them individually into BamHI/XhoI digested pNVQC5LSP-7.
The 1320 bp BamHI/XhoI **fragment containing the H6-p53** expression cassette from
pMM106 and pMM108 was inserted into pNVQC5LSP-7 to yield pMM110 and pMM112,
respectively, while the 1317 bp BamHI/XhoI fragment derived from pMM107 and
inserted into pNVQC5LSP-7 yielded pMM111.

Detailed Description Text - DETX (651):

A **fragment containing the H6 promoted 5' end of the p53** gene fused to the 3' end of the p53 gene was generated by several PCRs as described below.

Detailed Description Text - DETX (654):

PCRIII: Plasmid p11-4 was used as template with oligonucleotides MM084 (SEQ ID NO:212) 5' CAGAAGCTACTACTACTACTACCCACCTGCACAAGCGCC 3' and MM085 (SEQ ID
NO:213) 5' AACTACTGTCCCGGGATAAAAATCAGTCTGAGTCAGGCCCCAC 3' to generate a 301 bp
fragment. The 301 bp PCR-derived **fragment contains the 3' end of the p53** gene, and the 5' end overlaps the 3' end of the PCRII product. MM084 (SEQ ID NO:212) primes from position 916 of the murine p53 gene toward the 3' end. MM085 (SEQ ID NO:213) primes from position 1173 toward the p53 gene 5' end. The three PCR products were pooled and primed with MM080 and MM085. The resultant 588 bp fragment contains a BamHI site followed by the H6 promoted 5' end of the p53 gene fused to the p53 gene 3' end followed by a SmaI site; the 5' end of the p53 gene ends at the XhoI site at position 37, and the 3' end starts at the SacII site at position 990 (FIG. 38). The 588 bp PCR-derived fragment was digested with BamHI and SmaI generating a 565 bp fragment which was inserted into BamHI/SmaI digested pNC5LSP5 (described below). The resultant plasmid, designated pMM136, was digested with KspI and XhoI to remove a 149 bp fragment, and the 953 bp KspI/XhoI fragment from p11-4 was inserted. The resultant plasmid, pMM148, contains the H6 promoted wild-type murine p53 in the ALVAC C5 insertion locus.

Detailed Description Text - DETX (663):

INSERTION OF MUTANT FORMS OF **HUMAN P53** INTO ALVAC AND NYVAC

Detailed Description Text - DETX (664):

Mutant forms of **Human p53** into ALVAC

Detailed Description Text - DETX (665):

FIG. 18 (Example 15) presented the sequence of the vaccinia H6 promoted **human wild type p53** gene cassette in an ALVAC-based recombinant, vCP207. In this example, to facilitate description of the mutant forms of the **human p53** gene being described, FIG. 39 (SEQ ID NO:215) presents only the coding sequence for the **human wild type p53** gene. The start codon is at position 1 and the stop codon is at position 1180.

Detailed Description Text - DETX (666):

Plasmid C.times.22A, containing a mutant form of the **human p53** gene, was received from Arnold Levine (Princeton University, Princeton, N.J.). Relative to the wild type p53 sequence presented in FIG. 39, the G at nucleotide position 524 is substituted with an A, changing the arg amino acid at codon 175 of the wild type protein to a his amino acid in C.times.22A.

Detailed Description Text - DETX (667):

Plasmid pMM110 (Example 15, FIG. 18) contains the vaccinia H6 promoted wildtype **human p53** gene in the ALVAC C5 insertion site. The **human p53** gene contains two Pflml sites. p53 coding sequences upstream from the first Pflml site and downstream from the second Pflml site are the same in pMM110 as in C.times.22A. pMM110 was digested with Pflml to remove the 853 central base pairs of the p53 gene. The 853 bp Pflml fragment from C.times.22A containing the base change at position 524 was inserted. The resultant plasmid, pMM143, contains the H6 promoted mutant p53 gene.

Detailed Description Text - DETX (668):
Recombination between donor plasmid pMM143 and ALVAC rescuing virus generated recombinant virus vCP270. vCP270 contains the mutant form of the **human p53** gene under the control of the vaccinia H6 promoter in the C5 locus.

Detailed Description Text - DETX (669):
Plasmid pR4-2 containing a mutant form of the **human p53** gene was received from Arnold Levine (Princeton University, Princeton, N.J.). Relative to the wild type p53 sequence presented in FIG. 39, the G at nucleotide position 818 is substituted by an A, changing the arg codon at amino acid position 273 to a his codon in pR4-2.

Detailed Description Text - DETX (670):
Plasmid pMM110 (Example 15, FIG. 18) contains the vaccinia H6 promoted **human wildtype p53** gene in the ALVAC C5 insertion site. p53 coding sequences upstream from the first Pflml site and p53 coding sequences downstream from the second Pflml site are the same in pMM110 as in pR4-2. pMM110 was digested with Pflml to remove the 853 central base pairs of the p53 gene. The 853 bp Pflml fragment from pR4-2 containing the base change at nucleotide position 818 was inserted. The resultant plasmid, pMM144, contains the H6 promoted mutant form of the **human p53** gene in the C5 insertion locus.

Detailed Description Text - DETX (671):
Recombination between donor plasmid pMM144 and ALVAC rescuing virus generated recombinant virus vCP269. vCP269 contains the mutant form of the **human p53** gene under the control of the vaccinia H6 promoter in the C5 locus.

Detailed Description Text - DETX (672):
Mutant forms of **Human p53** into NYVAC

Detailed Description Text - DETX (673):
Plasmid C.times.22A, described above, contains a mutant form of the **human p53** gene, in which the G at nucleotide position 524 (FIG. 39) is substituted by an A, changing the arg codon at amino acid position 175 to a his codon in C.times.22A.

Detailed Description Text - DETX (674):

Plasmid pMM106 (Example 15) contains the vaccinia H6 promoted wild-type **human p53** gene in the NYVAC I4L insertion locus. p53 coding sequences upstream from the first PflmI site and p53 coding sequences downstream from the second PflmI site are the same in pMM106 as in C.times.22A. pMM106 was digested with PflmI to remove the 853 central base pairs of the p53 gene. The 853 bp PflmI fragment from C.times.22A containing the base change at position 524 was inserted. The resultant plasmid, pMM140, contains the H6 promoted mutant p53 gene.

Detailed Description Text - DETX (675):

Recombination between donor plasmid pMM140 and NYVAC rescuing virus generated recombinant virus vP1234. vP1234 contains the mutant form of the **human p53** gene under the control of the vaccinia H6 promoter in the I4L locus.

Detailed Description Text - DETX (676):

Plasmid pR4-2, described above, contains a mutant form of the **human p53** gene, in which the G at nucleotide position 818 (FIG. 39) is substituted by an A, changing the arg codon at amino acid position 273 to a his codon in pR4-2.

Detailed Description Text - DETX (677):

pMM106 (Example 15) contains the H6 promoted wild-type **human p53** gene in the I4L locus. p53 coding sequences upstream from the first PflmI site and p53 coding sequences downstream from the second PflmI site are the same in pMM106 as in pR4-2. pMM106 was digested with PflmI to remove the 853 central base pairs of the p53 gene. The 853bp PflmI fragment from pR4-2 containing the base change at position 818 was inserted. The resultant plasmid, pMM141, contains the H6 promoted mutant p53 gene.

Detailed Description Text - DETX (678):

Recombination between donor plasmid pMM141 and NYVAC rescuing virus generated recombinant virus vP1233. vP1233 contains the mutant form of the **human p53** gene under the control of the vaccinia H6 promoter in the I4L locus.

Detailed Description Text - DETX (679):

A listing of the wildtype and mutant forms of murine **p53 and the mutant forms of human p53** present in ALVAC and NYVAC recombinants described in Examples 31 and 32 is provided in Table 29.

Detailed Description Text - DETX (681):

ALVAC and NYVAC based recombinants vP1101, vP1096, vP1098, vCP207, vCP193, vCP191 (all described in Example 15; Table 22, as well as ALVAC and NYVAC based recombinants vCP270, vCP269, vP1233, vP1234 described in this Example, Table 29), contain wild type or mutant forms of the **human p53** gene. All of these recombinant virus were assayed for expression of the **human p53** gene using immunoprecipitation.

Detailed Description Text - DETX (682):

Recombinant or parental virus were inoculated onto preformed monolayers of tissue culture cells in the presence of radiolabelled .sup.35 S-methionine and treated as previously described (Taylor et al., 1990). Immunoprecipitation reactions were performed using a **human p53** specific monoclonal antibody 1801. A protein of between 47 and 53 kDa was precipitated from cells infected with any of the recombinant viruses, vP1101, vP1096, vP1098, vCP207, vCP193, vCP191, vCP270, vCP269, vP1233, or vP1234, but not from uninfected cells or cells infected with parental ALVAC or NYVAC virus.

Detailed Description Paragraph Table - DETL (29):

TABLE 29 _____ Recombinant Virus Parent Virus Species Gene Insert _____ vCP263 ALVAC murine w.t. p53 vCP267 ALVAC murine p53 (+3 aa) VCP27O ALVAC **human p53** (aa 175; R to H) vCP269 ALVAC **human p53** (aa 273; R to H) vP1234 NYVAC **human p53** (aa 175; R to H) vP1233 NYVAC **human p53** (aa 273; R to H)

_____

US-PAT-NO: 5756455

DOCUMENT-IDENTIFIER: US 5756455 A

TITLE: Amplification of human MDM2 gene in human tumors

DATE-ISSUED: May 26, 1998

INVENTOR-INFORMATION:
NAME	CITY	STATE	ZIP CODE	COUNTRY
Kinzler; Kenneth W.	Baltimore	MD	N/A	N/A
Vogelstein; Bert	Baltimore	MD	N/A	N/A

APPL-NO: 08/ 390515

DATE FILED: February 17, 1995

PARENT-CASE:

   This application is a divisional application of Ser. No. 08/044,619, filed Apr. 7, 1993, now U.S. Pat. No. 5,420,263, which is a continuation-in-part of Ser. No. 07/903,103, filed Jun. 23, 1992, now U.S. Pat. No. 5,411,860, which is a continuation-in-part of Ser. No. 07/867,840, filed Apr. 7, 1992, now abandoned.

US-CL-CURRENT: 514/12, 530/324 , 530/350

ABSTRACT:

   A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and allows the cell to escape from p53-regulated growth.

3 Claims, 24 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 17

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Abstract Text - ABTX (1):
   A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated

hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and allows the cell to escape from p53-regulated growth.

Brief Summary Text - BSTX (6):

While there exists an enormous body of evidence linking **p53 gene mutations to human** tumorigenesis (Hollstein et al., 1991, Science 253:49-53) little is known about cellular regulators and mediators of p53 function.

Brief Summary Text - BSTX (7):

Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated ras oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253:49-53. The transformed fibroblasts were found to produce elevated levels of **human p53** protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Brief Summary Text - BSTX (8):

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type **human p53** gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

Brief Summary Text - BSTX (9):

Hinds et al., supra also expressed **human p53** protein in transformed rat cells. When the expressed **human p53** was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified $M.sub.r$ 90,000 protein was coimmunoprecipitated. This suggested that the rat $M.sub.r$ 90,000 protein is in a complex with the **human p53** protein in the transformed rat cell line.

Brief Summary Text - BSTX (19):

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (22):

Still another object of the invention is to provide polypeptides which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (23):

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to **human p53** in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

Drawing Description Text - DRTX (7):

FIG. 6 shows the determination of MDM2 and p53 domains of interaction. FIG. 5A and FIG. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (exA-responsive .beta.-galactosidase reporter). Yeast clones expressing .beta.-galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined. .beta.-galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. FIG. 6C. Random **fragments of p53** were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Detailed Description Text - DETX (13):

It has been found that amino acid residues 13-41 of **p53** (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and **p53**. However, additional residues on either the amino or carboxy **terminal** side of the **peptide** appear also to be required. Nine to 13 additional **p53** residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from **p53**-regulated growth control in sarcomas, the use of **p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53**-regulated growth control.

Detailed Description Text - DETX (14):

Suitable **p53-derived peptides** for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

Detailed Description Text - DETX (15):

DNA encoding the MDM2-binding, **p53-derived peptide,** or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus, or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding

peptide. The DNA can be administered, inter alia encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of **peptide, the goal is t   alleviate the sequestrati n of p53** by MDM2.

Detailed Description Text - DETX (18):

   The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to **human p53**. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

Detailed Description Text - DETX (27):

   To determine whether the hMDM2 protein could bind to **human p53** protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in FIG. 1 (SEQ ID NO:2) from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Detailed Description Text - DETX (33):

   The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (FIG. 2, lanes 2 and 3). However, when in vitro translated **human p53** was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association in vitro (FIG. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (FIG. 2, lanes 8 and 9). When an in vitro translated mutant form of p53 (175.sup.his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

Detailed Description Text - DETX (58):

   This assay was then applied to mapping the interaction domains of each protein. Full length cDNA **fragments encoding MDM2 or p53** were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein. METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert

was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D. M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 lexA-responsive .beta.-galactosidase reporter). Approximiately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence.
MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent clones are diagrammed in FIG. 6A. The MDM2 sequences of the remaining 15 **p53-dependent clones coded for peptides** ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of FIG. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Detailed Description Text - DETX (59):
 Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-**terminal** to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three **p53** sequences shown in FIG. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated **fragments**.

Detailed Description Text - DETX (60):
 The resultant yeast colonies were examined for .beta.-galactosidase activity in situ. Of approximately 5000 clones containing MDM2 **fragments** fused to the lexA DNA binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of .beta.-galactosidase (about 5-fold less than the other fifteen clones) and .beta.-galactosidase expression was independent of **p53** expression (FIG. 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable .beta.galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino **terminal** region of MDM2 (FIG. 6B). The .beta.-galactosidase activity of these clones was dependent on

p53 co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length **p53** contained MDM2 codons 1 to 118 (FIG. 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated **fragments** of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

Detailed Description Text - DETX (61):

In a converse set of experiments, yeast clones containing **fragments of p53** fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (FIG. 2C);SEQ ID NO:1. The minimal overlap between these three **fragments** contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the **fragments** required 9-12 amino acids on either side of codons 13-41 for activity (FIG. 6C). To further test the idea that the amino termainal region of **p53** was required for MDM2 binding, we generated an additional yeast strain expressing the lex-DNA binding domain fused to **p53** codons 74-393) and the B42 acidic activation domainfused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-**terminus of p53** were required for the interaction.

Detailed Description Text - DETX (62):

Sequence analysis showed that all **p53 and MDM2 fragments** noted in FIG. 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in FIG. 6 expressed the relevant proteins at similar levels, as shown by Western blotting (FIG. 7).

Claims Text - CLTX (2):

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting of a **portion of p53,** wherein said polypeptide comprises amino acids 1-50 of p53 as shown in SEQ ID NO:1, said polypeptide being capable of binding to human MDM2 as shown in SEQ ID NO:3.

Claims Text - CLTX (4):

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting of a **portion of p53, said portion comprising amino acids 13-41 of p53** as shown in SEQ ID NO:1 and at least nine additional **p53** residues on either the amino or carboxy **terminal** side, wherein said polypeptide lacks the homo-oligomerization domain of **p53, and said polypeptide is capable of binding to human** MDM2 as shown in SEQ ID NO:3.

Claims Text - CLTX (6):

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting of a **porti n of p53, said portion comprising amin acids 13-41 f p53** as shown in SEQ ID NO:1 and at least nine additional **p53** residues on either the amino or carboxy **terminal** side, said polypeptide being capable of binding to human MDM2 as shown in SEQ ID NO:3, wherein said polypeptide lacks amino acids 138-393 of p53as shown in SEQ ID NO:6, 7, 8, or 9 and said polypeptide is capable of binding to human MDM2.

Other Reference Publication - OREF (2):

Hinds, et al., "Mutant **p53 DNA Clones From Human** Colon Carcinomas Cooperate With Ras in Transforming Primary Rat Cell: A Comparison of the Hot Spot Mutant Phenotypes", Cell Growth & Differentiation, 1:561-580 (1990).

Other Reference Publication - OREF (7):

Leach, et al., "**p53 Mutation and MDMS Amplification in Human** Soft Tissue Sarcomas", Cancer Research 53:2231-2234 (1993).

US-PAT-NO: 5747650

DOCUMENT-IDENTIFIER: US 5747650 A

TITLE: P53AS protein and antibody therefor

DATE-ISSUED: May 5, 1998

INVENTOR-INFORMATION:
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APPL-NO: 08/ 644456

DATE FILED: May 10, 1996

PARENT-CASE:

   This is a continuation-in-part of U.S. patent application Ser. No.
08/106,496, filed Aug. 2, 1993.

US-CL-CURRENT: 530/387.7, 530/387.1 , 530/388.8 , 530/389.1 , 530/389.2

ABSTRACT:

   In accordance with the present invention, we have discovered and purified a
protein designated herein as p53as, which protein is present in normal cells of
a mammal and is essentially identical to known normal growth controlling
protein p53 of the same mammal, at least until the final 50 amino acids of the
carboxy terminal end of the protein. The invention further includes an
antibody specific for protein p53as, which antibody is designated herein as Ab
p53as. The antibody may be either a monoclonal or polyclonal antibody and may
be specific for p53as of any particular mammal such as mice and humans.

11 Claims, 26 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 11

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Brief Summary Text - BSTX (3):
   The p53 gene which encodes for p53 protein is defective in over half of all
human cancers. It is furthermore significant because introduction of a normal
p53 gene into a variety of cancer cells arrests their growth. Thus, defects in
the p53 gene product (that is, the p53 protein) are common in many cancers and,
if corrected, could inhibit cancer cell growth. In many **human cancers, the p53**

protein is inactive because of mutation of the p53 gene. Replacement of a single amino acid can be sufficient to change the normal folding of the p53 protein, making it inactive as a growth control gene. In certain cells, the folding of a mutant p53 protein can be stabilized in the normal conformation by binding to cellular factors, suggesting that it may be possible to create **peptides which bind to p53** protein and cause it to be maintained in the normal conformation (conformations are forms of a protein created due to folding; conformations can change without (or with) changes in amino acid sequence). The normal conformation has the tumor suppressor effect. Cells expressing primarily mutant p53 conformation give rise to aggressive tumors at high frequency while cells which primarily express p53 protein in a normal conformation give rise to slow-growing tumors at low frequency.

Drawing Description Text - DRTX (4):

FIG. 3 is a domain map of **p53** protein showing changes introduced by alternative splicing. Mouse **p53** has 390 amino acids. Domains (see Vogelstein et al., (1992), "**p53** function and dysfunction", Cell 70, 523-526 and references therein) are ACT: transcriptional activation domain; HSP; heat shock protein binding region of mutant **p53**; HOT spots; highly conserved regions among **p53** proteins in which most transforming mutations occur; PAb240; region binding antibody conformation-specific for certain mutants, murine amino acids 156-214; PAb246: region binding antibody to normal wt. conformation, murine amino acids 88-109; PAb421: region binding antibody to wt. and mutant conformation, amino acids 370-378; NUC:nuclear localization signal; CDC2 **kinase** serine **phosphorylation** site; CK2 casein **kinase** serine **phosphorylation** site, which is also the site of 5.8rRNA binding; OLIGO: site of **p53** self-association. The expected changes in the C-**terminal** region of protein translated from alternatively spliced wt. (Han et al., (b) (1992), supra) or mutant **p53** mRNA (Arai et al., (1986) "Immunologically distinct **p53** molecules generated by alternative splicing", Mol. and Cell. Biol., 6, 3232-3239) are shown. The segment of intron 10 retained in p53as mRNA is indicated as a triangle between exons. Acidic amino acids (within a predicted alpha-helix spanning 334-356) and basic amino acids (between position 363 and 386--underlined in the C-**terminal peptide** sequence at bottom) are labeled according to Sturzbecher et al. (1992), "A C-**terminal** a-helix plus basic region motif is the major structural determinant of **p53** tetramerization", Oncogene 7, 1513-1523.

Detailed Description Text - DETX (7):

Polyclonal and monoclonal antibodies to mouse p53 available prior to the present invention do not specifically recognize p53as. These include PAb246 which recognizes mouse p53 in its normal folding state, PAb240 which recognizes certain mutant p53 proteins, PAb421 which recognizes the carboxyl terminal amino acids replaced or lost in p53as. Similarly, **human p53** is recognized by monoclonal and polyclonal antibodies. No report of a **human p53** generated by alternative splicing at the carboxyl terminus has been reported and no specific antibody to human p53as is available.

Detailed Description Text - DETX (10):

Alternative splicing of mouse p53 RNA results in insertion of 96nt from

intron 10 of the p53 gene. These 96nt encode (in frame) 17 amino acids which are distinct from those in the major p53 RNA form, beginning at residue 365 and extending to residue 381, followed by a stop codon which results in truncation by 9 amino acids. This 17 amino acid **peptide f alternatively spliced mouse p53, called mous p53as peptide** is: LQPRAFQALIKEESPNC. It was produced by standard synthesis, tested for authenticity and is stored in the laboratory. Details and procedures are as follows:

Detailed Description Text - DETX (27):

   Human p53as protein is defined herein as the **human p53** protein 1) which is generated from a **p53 transcript detectable in human** cells by reverse transcriptase (RT)/polymerase chain reaction (PCR) (as described below) which is itself generated by alternative splicing of a region of intron 10 of the **human p53** gene, and 2) which contains carboxyl **terminal** amino acids distinct from those of the major **human p53** protein. (Singular is used but is not meant to rule out the possibility that more than one p53as protein is made in human cells). Antibodies to human p53as **peptide** permit verification of the presence of p53as in human cells.

Detailed Description Text - DETX (28):

   Prior to the present invention, no human p53as protein in normal cells (alternatively spliced at the carboxyl **terminus,** analogous to mouse p53as) has been reported or suggested. The mouse and **human p53** cDNA sequences are 81% identical and have functional domains in common. There are three lines of evidence pointing to the existence of human p53as. First, two PCR products have been amplified by RT/PCR from human cDNA using primers which span intron 10 created from mouse exon 10 and exon 11 sequences. Second, two **p53** proteins are detectable by molecular weight differences in western immunoblots or immunoprecipitations using polyclonal antibodies to **human p53,** for example, by Gupta et al. (Proc. Natl. Acad. Sci. 90: 2817-2921, 1993) who used antibody CM-1 and protein from Hodgkins disease tumor cells. This has been attributed to either distinct **phosphorylation** states or a polymorphism at amino acid 72. CM-1 is expected to react with multiple regions on the **p53** gene and thus would be expected to react with both **human p53** and p53as proteins. Thus the presence of human p53as could account for the data in the literature demonstrating two **p53** proteins distinguishable by molecular weight. Third, human intron 10 encodes a **peptide** which has a motif (SPPC) similar to the last 4 amino acids of the mouse p53as (SPNC).

Detailed Description Text - DETX (30):

   Primers are constructed which are used to amplify by polymerase chain reaction (PCR) a region of the **human p53** cDNA including part of exon 10, all of intron 10 sequences retained in the alternatively spliced **p53** mRNA and part of exon 11. Human cDNA is generated from cellular RNA (isolated by guanidinium/cesium chloride extraction) by RT/PCR. RT/PCR is carried out as follows: 5 .mu.g of human cell total RNA is combined with 1 mM each of 4 deoxynucleotidetriphosphates, 5 .mu.g random hexamer primer (to make cDNA to all available mRNA), 5 .mu.AMV reverse transcriptase (RT, 5 to 10 units per .mu.l), 3.5 mM MgCl.sub.2 (or as optimized), 2.5 .mu.l RNasin 5 .mu.l PCR

buffer (Perkin Elmer; without Mg.sup.2+) and depc-treated water to adjust the volume to 50 .mu.l. Reaction is allowed to proceed at 23.degree. C. for 10 minutes, 42.degree. C. for 1h and 95.degree. C. for 10 minutes then transferred to ice. An additional 0.2 .mu.l of RT is added and the reaction is repeated 1X. 1 .mu.l of the RT reaction product mix is used to provide the cDNA templates for **human p53** and p53as C**-terminal** regions for amplification by PCR. PCR is optimized to obtain efficient production of the specific product and minimize background. PCR is performed for 35 cycles of denaturation (95.degree. C., 30 sec), annealing (60.degree. C., 1min) and extension (72.degree. C, 3 min) in a DNA thermal cycler. Amplified **fragments of human p53** and p53as C**-terminal** coding regions are desalted by centricon ultrafiltration, digested with the restriction enzymes appropriate to the synthetic primers (see example below) and isolated from low melting temperature agarose for cloning into pGEM3zf(+) (Promega) for the sense strand or pBluescript KS(+) (Stratagene) for the antisense strand and transfected into E. coli for production and sequencing as we have described (Han et al. supra). Human cells as the source of RNA include (but are not limited to) normal human epidermal keratinocytes and two clones (B and F2A) of squamous cell carcinoma line SCC-12. The PCR amplification product generated using the primers which span intron 10 include the major **p53** transcript and p53as transcript(s). These are distinguished by differences in molecular weight and/or by sequencing of the amplified PCR products as has been demonstrated previously for mouse p53as transcripts (Han et al. (b) supra). Sequencing of the PCR products permits determination of the sequence of the protein encoded by human p53as RNA. The amino acid sequence of the human p53as protein is compared to that of the major **human p53** protein to determine the unique sequence at the carboxyl **terminal** region of human p53as protein.

Detailed Description Text - DETX (31):

   An example of a primer set which spans intron 10 of the **human p53** gene is:
5' primer/sense strand ATCGAAGCTTGAGATGTTCCGAGAGAGCTGAAT (within exon 10 beginning at nucleotide 17,593 of the genomic p53 sequence Genbank accession No. X54156, with additional nucleotides added to the 5' end, ATCG and restriction endonuclease site HindIII to facilitate cloning and sequencing--underlined) and 3' primer antisense strand ATCGTCTAGAGCTTCTGACGCACACCTATTG (within exon 11 beginning at nucleotide 18794 in the 5' to 3' direction to nucleotide 18774, with ATCG and XbaI restriction endonuclease site added--underlined).

Detailed Description Text - DETX (33):

   Polyclonal antibody to mouse p53as unique peptide noted above has been raised in rabbits, its high titer has been determined by enzyme linked immunosorbent assay (ELISA), its specificity for p53 protein has been determined by immunoprecipitation from mouse cells, western immunoblotting of anti-p53 precipitable protein to a polyclonal antibody to p53 (CM5, reactive with epitopes shared by **p53 and p53as proteins), ability of the peptide** to competitively block reactivity in cells and in western immunoblots, and the ability of the p53as peptide to block binding of p53as antibody but not block the binding other p53 antibodies (PAb421 and pAb246) which bind to epitopes distinct from the unique region of p53as.

Detailed Description Text - DETX (107):

In addition to alterations in the oligomerization domain predicted from the above studies, p53as has lost the casein kinase II phosphorylation/5.8s rRNA binding site located at serine 389 (see FIG. 1). Loss of the phosphorylation site at serine-389 by mutation negates p53 anti-proliferative activity (Milne et al., (1992), "Mutation of the casein kinase II phosphorylation site abolishes the anti-proliferative activity of p53 ", Nucleic Acids Res. 20, 5565-5570; Bischoff et al., (1992), "**Human p53** inhibits growth in Schizosaccharomyces pombe", Mol. and Cell. Biol. 12, 405-411; Nigro et al., (1992), "**Human p53** and CDC2Hs genes combine to inhibit the proliferation of Saccharomyces cerevisiae", Mol. and Cell. Biol. 12, 1357-1365). Hupp et al., supra, have shown that factors acting at the C-terminus are important for activation of the DNA binding capacity of wt. p53, including: phosphorylation at the conserved C-terminal serine, PAb421 antibody binding to its carboxyl terminal epitope, and proteolysis or engineered loss of the last 30 carboxyl terminal amino acids. In addition, regions of the p53 protein which mediate binding to other cellular proteins such as heat shock protein (Hainaut et al., supra) or mdm2 (Momand et al. (1992), "The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53 -mediated transactivation", Cell 69, 1237-1245), conceivably could be directly or indirectly altered.

Detailed Description Text - DETX (114):

Mouse monoclonal antibodies to **p53** were PAb421, PAb240 and PAb246 (Oncogene Science, Uniondale, N.Y.). Isotype IgG.sub.2a (PAb421 and PAb240) and IgG.sub.1 (PAb246, Becton/Dickinson, Mountain View, Calif.) were used as sera controls. Rabbit polyclonal antibody CM5 was a gift from Dr. David Lane. Anti-**peptide** antibody to the **terminal** 17 amino acids unique to p53as (see FIG. 1B) was generated as follows: The 17 amino acid **peptide** to p53as was synthesized by the RPCI Biopolymer Facility and determined to be 90 to 95% pure by HPLC and mass spectroscopy and accurate by amino acid sequencing. Following collection of pre-immune serum, New Zealand White female rabbits were immunized by intradermal injection of 500 .mu.g **peptide** plus Freund's complete adjuvant (FCA) at multiple sites, concurrent with intramuscular injection of Pertussis vaccine (RPCI Springville Laboratories). After 3 weeks, an additional 250 .mu.g of **peptide** was administered with Freund's incomplete adjuvant (FIA) at weekly intervals for 3 weeks. The p53as anti-**peptide** serum was affinity-purified by coupling 4.4 mg p53as **peptide** to an AminoLink column (Pierce, Rockford, Ill.) according to manufacturers instructions. Ammonium sulfate (40%)-precipitated pre-immune serum was used as a control. Competition assays were performed by incubation of antibodies (1:1 ration by weight) with p53as **peptide** (sequence shown in FIG. 1) or an unrelated **peptide** (sequence: GRNDCIIDKIRRKNCD) for 2 h at room temperature prior to the immunoreaction with cells or **peptide** in ELISA assays.

Detailed Description Text - DETX (116):

Nunc-Immuno MaxiSorb 96 well plates (Nunc, Denmark) were coated with 50 ng/well p53as **peptide** in 15 mM sodium carbonate buffer, pH 9.6. After blocking with 2% BSA (KPL, Gaithersburg, Md.) in PBS at 37.degree. C. for 1 h, anti-**p53** monoclonal antibodies, anti-p53as antibody (affinity-purified to **peptide**) or

pre-immune serum control (pre-I) were diluted 1/50 to 1/640,000 and added to the wells in 100 .mu. volume. Secondary antibody was peroxidase-conjugated goat anti-rabbit immunoglobulin (DAKO, Carpinteria, Calif.) at 1/1000. TMB peroxidase substrate system solution (KPL, Gaithersburg, Md.) was added and color development was **terminated** after 4 minutes using 4M H.sub.2 SO.sub.4. Absorbance at 450 nm was detected using a BioTek plate reader (Winooski, Vt.).

Detailed Description Text - DETX (124):

RNA was isolated from cells approximately 70 to 100% confluent by guanidinium/cesium chloride extraction and dissolved in diethylpyrocarbonate-treated water for northern blot analysis as described previously (Han et al. (1990), "Altered levels of endogenous retrovirus-like sequence (VL30) RNA during mouse epidermal cell carcinogenesis", Mol. Carcinogenesis 3:75-82). A 500 base pair PstI **fragment of p53**-422 was used for p53 detection (Oren et al. (1983), "Molecular cloning of a cDNA specific for the murine p53 cellular tumor antigen", Proc. Natl. Acad. Sci. USA, 80, 56-59) and as 840 bp EcoRI-SalI fragment of pA6 was used for 7S RNA detection as a control for RNA loading (Balmain et al., (1982), "Cloning and characterization of the abundant cytoplasmic 7S RNA from mouse cells", Nucleic Acids, Res., 10,4259-4277). Probes were labeled with [.varies.-32P]dCTP by the random primer method using a multiprime labeling kit (Amersham, Arlington Heights, Ill.). 32P-labeled probe was used at a final concentration of 1 to 2.times.10.sup.6 cpm/ml. Differences in p53 RNA abundance were quantitated by densitometry of exposed films (Fastscan computing densitometer, Molecular Dynamics, Sunnyvale, Calif.) after adjustment for 7S RNA.

Other Reference Publication - OREF (4):

Bischoff, J.R., et al. (1992) **Human p53** Inhibits Growth in Schizosaccharomyces Pombe. Mol. and Cell. Biol. 12, 1405-1411.

Other Reference Publication - OREF (6):

Crook, T., et al. (1991) Modulation of Immortalizing Properties of **Human Papillomavirus Type 16E7 by p53** Expression. J. Virol. 6, 505-510.

Other Reference Publication - OREF (33):

Nigro, J.M., et al. (1992) **Human p53** and CDC2Hs Genes Combine to Inhibit the Proliferation of Saccharomyces Cerevisiae. Mol. and Cell Biol. 12, 1357-1365.

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TITLE: Methods and compositions comprising DNA damaging agents
                and p53

DATE-ISSUED: May 5, 1998

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APPL-NO: 08/ 233002

DATE FILED: April 25, 1994

PARENT-CASE:

   The present application is a continuation-in-part of co-pending U.S. patent
application Ser. No. 08/145,826, filed Oct. 29, 1993; which is a
continuation-in-part of U.S. patent application Ser. No. 07/960,513, filed
Oct. 13, 1992; which is a continuation-in-part of U.S. Ser. No. 07/665,538,
filed Mar. 6, 1991 now abandoned; the entire text and figures of which
disclosures are incorporated herein by reference without disclaimer.

US-CL-CURRENT: 514/44, 435/320.1 , 435/375 , 514/2

ABSTRACT:

   The present invention relates to the use of tumor suppressor genes in
combination with a DNA damaging agent or factor for use in killing cells, and
in particular cancerous cells. A tumor suppressor gene, p53, was delivered via
a recombinant adenovirus-mediated gene transfer both in vitro and in vivo, in
combination with a chemotherapeutic agent. Treated cells underwent apoptosis
with specific DNA fragmentation. Direct injection of the p53-adenovirus
construct into tumors subcutaneously, followed by intraperitoneal
administration of a DNA damaging agent, cisplatin, induced massive apoptotic
destruction of the tumors. The invention also provides for the clinical
application of a regimen combining gene replacement using replication-deficient
wild-type p53 adenovirus and DNA-damaging drugs for treatment of human cancer.

105 Claims, 45 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 22

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Drawing Description Text - DRTX (5):
   FIG. 2B. Agarose gel analysis of PCR products. Two pairs of primers that define 1.4-kb **(p53) and 0.86-kb (Ad5) DNA fragments** were used in each reaction. DNA templates used in each reaction were pEC53 plasmid (lane 1), Ad5/RSV/GL2 DNA (lane 2), no DNA (lane 3), and Ad5CMV-p53 DNA (lane 4). The lane labeled (M) corresponds to molecular weight markers.

Detailed Description Text - DETX (11):
   Casey and colleagues have reported that transfection of DNA encoding wild-type **p53 into two human** breast cancer cell lines restores growth suppression control in such cells (Casey et al., 1991). A similar effect has also been demonstrated on transfection of wild-type, but not mutant, **p53 into human** lung cancer cell lines (Takahasi et al., 1992). The p53 appears dominant over the mutant gene and will select against proliferation when transfected into cells with the mutant gene. Normal expression of the transfected p53 does not affect the growth of cells with endogenous p53. Thus, such constructs might be taken up by normal cells without adverse effects.

Detailed Description Text - DETX (28):
   The present invention provides cancer gene therapy with a new and more effective tumor suppressor vector. This recombinant virus exploits the advantages of adenoviral vectors, such as high titer, broad target range, efficient transduction, and non-integration in target cells. In one embodiment of the invention, a replication-defective, helper-independent adenovirus is created that expresses wild type p53 (Ad5CMV**-p53) under the control of the human** cytomegalovirus promoter.

Detailed Description Text - DETX (31):
   The design and propagation of the preferred p53 adenovirus is diagramed in FIG. 1. In connection with this, an improved protocol has been developed for propagating and identifying recombinant adenovirus (discussed below). After identification, the p53 recombinant adenovirus was structurally confirmed by the PCR analysis, as indicated in FIG. 2. After isolation and confirmation of its structure, the **p53 adenovirus was used to infect human** lung cancer cell line H358, which has a homozygous p53 gene deletion. Western blots showed that the exogenous p53 protein was expressed at a high level (FIG. 4 and FIG. 5) and peaked at day 3 after infection (FIG. 6).

Detailed Description Text - DETX (32):
   It was also shown in a p53 point mutation cell line H322 that the mutant p53 was down regulated by the expression of the exogenous p53. As an experimental

control, a virion (Ad5/RSV/GL2) that had a structural similarity to that of Ad5CMV-p53 was used. This virion contained a luciferase CDNA driven by Rous sarcoma virus LTR promoter in the expression cassette of the virion. Neither p53 expression nor change in actin expression was detected in cells infected by the virion Ad5/RSV/GL2. Growth of the H358 cells infected with Ad5CMV-p53 was greatly inhibited in contrast to that of noninfected cells or the cells infected with the control virion (FIG. 7A). Growth of H322 cells was also greatly inhibited by the **p53 virion (FIG. 7B), while that of human** lung cancer H460 cells containing wild-type p53 was less affected (FIG. 7C).

Detailed Description Text - DETX (34):
    Tests in nude mice demonstrated that tumorigenicity of the Ad5CMV-p53-treated H358 cells was greatly inhibited. In a mouse model of orthotopic human lung cancer, the tumorigenic H226Br cells, with a point mutation in p53, were inoculated intratracheally 3 days prior to the virus treatment. Intratracheal instillation of Ad5CMV-p53 prevented tumor formation in this model system suggesting that the modified adenovirus is an efficient vector for mediating transfer and expression of tumor suppressor genes in **human cancer cells and that the Ad5CMV-p53** virus may be further developed into a therapeutic agent for use in cancer gene therapy.

Detailed Description Text - DETX (35):
    Ad5CMV-p53 mediated a high level of expression of the **p53 gene in human** lung cancer cells as demonstrated by Western blot analysis. Exogenous p53 protein was approximately 14 times more abundant than the endogenous wild-type p53 in H460 cells and about two to four times more abundant than the .beta.-actin internal control in H358 cells. The high level of expression may be attributed to (1) highly efficient gene transfer, (2) strong CMV promoter driving the p53 CDNA, and (3) adenoviral E1 enhancer enhancing the p53 CDNA transcription. The duration of p53 expression after infection was more than 15 days in H358 cells. However, there was a rapid decrease in expression after postinfection day 5. PCR analysis of the DNA samples from the infected H358 cells showed a decrease of the viral DNA level with the decreased protein level, indicating the loss of viral DNA during the continuous growth of cancer cells in vitro.

Detailed Description Text - DETX (83):
    Ad5CMV-p53-Directed **p53 Gene Expression in Human** Lung Cancer Cells

Detailed Description Text - DETX (84):
    This example describes the use of recombinant **p53 adenovirus to infect human** lung cancer cells with a homozygous p53 gene deletion. The results show that growth of these cells and expression of mutant p53 was suppressed, indicating the potential of the Ad5CMV-p53 virion as a useful agent for control of metastatic cells.

Detailed Description Text - DETX (88):
    Western blotting analysis was performed on total cell lysates prepared by lysing monolayer cells in dishes with SDS-PAGE sample buffer (0.5 ml per 60-mm

dish) after rinsing the cells with phosphate-buffered saline (PBS). For
SDS-PAGE analysis lanes were loaded with cell lysates equivalent to
5.times.10.sup.4 cells (10-15 ml). The proteins in the gel were transferred to
Hybond.TM.-ECL membrane (Amersham, Arlington Heights, Ill.). The membranes
were blocked with 0.5% dry milk in PBS and probed with the primary antibodies:
mouse anti-human p53 monoclonal antibody PAb 1801 and mouse anti-human
.beta.-actin monoclonal antibody (Amersham), washed and probed with the
secondary antibody: horseradish peroxidase-conjugated rabbit anti-mouse IgG
(Pierce Chemical Co., Rockford, Ill.). The membranes were developed according
to the Amersham's enhanced chemiluminescence protocol. Relative quantities of
the exogenous p53 expressed were determined by densitometer (Molecular Dynamics
Inc., Sunnyvale, Calif.).

Detailed Description Text - DETX (91):
    Growth of the H358 cells infected with Ad5CMV-p53 was greatly inhibited in
contrast to that of noninfected cells or the cells infected with the control
virion (FIG. 7A). Growth of H322 cells was also greatly inhibited by the p53
virion (FIG. 7B), while that of human lung cancer H460 cells containing wild
type p53 was affected to a lesser degree (FIG. 7C). Growth of the AdSCMV-p53
virus-infected H358 cells was inhibited 79%, whereas that of noninfected cells
or the cells infected with the control virus were not inhibited. Growth of
cell line H322, which has a point mutation in p53, was inhibited 72% by
Ad5CMV-p53, while that of cell line H460 containing wild-type p53 was less
affected (28% inhibition).

Detailed Description Text - DETX (113):
    The construction and identification of a recombinant adenovirus vector that
contains the CDNA that encodes human wt-p53 (Ad-p53) or luciferase (Ad-Luc)
were previously reported (Zhang, et al., 1993). Briefly, the p53 expression
cassette that contains human cytomegalovirus promoter, wt-p53 cDNA, and SV40
early polyadenylation signal, was inserted between the Xbal and Clal sites of
pXCJL.1. The p53 shuttle vector and the recombinant plasmid pJM17 were
cotransfected into 293 cells (Ad5-transformed human embryonic kidney cell line)
by a liposome-mediated technique. The culture supernatant of 293 cells showing
.the complete cytopathic effect was collected and used for subsequent
infections. The control Ad-Luc virus was generated in a similar manner.
Ad-p53 and Ad-Luc viruses were propagated in 293 cells. The presence of
replication competent virus was excluded by HeLa cell assays. The viral titers
were determined by plaque assays (Graham, et al., 1991).

Detailed Description Text - DETX (118):
    H358 cells were transduced in vitro with the human wt-p53 cDNA by exposure
to Ad-p53. Western blot analysis showed a high level of wt-p53 protein
expression as early as 24 hours after infection with Ad-p53, but no wt-p53 was
detected in parental (uninfected) cells or control cells infected with Ad-Luc
(data not shown). Concurrent immunohistochemical evaluation demonstrated
detectable wt-p53 protein in more than 80% of infected cells, suggesting that
the transfer and expression of p53 by AD-p53 was highly efficient (data not
shown).

Detailed Description Text - DETX (120):

An internucleosomal DNA ladder indicative of DNA fragmentation was evident in cells expressing wt-**p53** after 24 hours of exposure to CDDP; parental and Ad-Luc-infected cells, however, did not show DNA fragmentation (FIG. 11A). **Terminal** deoxynucleotidyl transferase (TdT)-mediated 2'-deoxyuridine-5'-triphosphate (dUTP)-biotin nick end labeling, which detects DNA fragmentation characteristic of apoptosis in situ, showed many apoptotic cells in Ad-**p53**-infected cells treated with CDDP for 24 hours as shown in FIG. 11 G which demonstrates darkly staining nuclei and nuclear **fragments** not present in FIGS. 11B-F.

Detailed Description Text - DETX (149):

Cai, D. W., Mukhopadhyay, T., Liu, T., Fujiwara, T., and Roth, J. A. Stable expression of the wild-type **p53 gene in human** lung cancer cells after retrovirus-mediated gene transfer. Human Gene Ther, 4:617-624, 1993.

Detailed Description Text - DETX (158):

Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Cai, D. W., Owen-Schaub, L. B., and Roth, J. A. A retroviral wild-type **p53 expression vector penetrates human** lung cancer spheroids and inhibits growth by inducing apoptosis. Cancer Res, 53:4129-4133, 1993.

Detailed Description Text - DETX (169):

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. (1991). **p53 mutations in human** cancers. Science 253:49-53.

Detailed Description Text - DETX (194):

Takahashi, T., Carbone, D., Takahashi, T., Nau, M. M., Hida, T., Linnoila, I., Ueda, R., and Minna, J. D. (1992). Wild-type but not mutant **p53 suppresses the growth of human** lung cancer cells bearing multiple genetic lesions. 1992. Cancer Res. 52:2340-2342.

Detailed Description Text - DETX (206):

Zhang, W. W., Fang, X., Mazur, W., French, B. A., Georges, R. N., and Roth, J. A. High-efficiency gene transfer and high-level expression of wild-type **p53 in human** lung cancer cells mediated by recombinant adenovirus. Cancer Gene Therapy, 1993.

Other Reference Publication - OREF (7):

Cheng, Jian, et al., "Suppression of Acute Lymphoblastic Leukemia by the **Human Wild-Type p53** Gene", Cancer Research, vol. 52, pp. 222-226, Jan. 1, 1992.

Other Reference Publication - OREF (23):

Mercer, W. Edward, et al., "Negative growth regulation in a glioblastoma

tumor cell line that conditionally expresses **human wild-type p53**", Proc. Natl. Acad. Sci. USA, vol. 87, pp. 6166-6170, Aug. 1990.

Other Reference Publication - OREF (25):
   Nigro, Janice M., et al., "Mutations in the **p53 gene occur in diverse human** tumour types", Nature, vol. 342, pp. 705-708, Dec. 7, 1989.

Other Reference Publication - OREF (33):
   Shay, Jerry W., et al., "A Role for Both RB and **p53 in the Regulation of Human** Cellular Senescence", Experimental Cell Research, vol. 196, pp. 33-39, 1991.

Other Reference Publication - OREF (38):
   Ullrich, Stephen J., et al., "**Human wild-type p53** adopts a unique conformational and phosphorylation state in vivo during growth arrest of glioblastoma cells", Oncogene, vol. 7, pp. 1635-1643, 1992.

Other Reference Publication - OREF (90):
   Rau et al., "Response of **p53 to treatment with actinomycin D in human** mammary carcinoma cell lines," Journal of Cancer Research and Clinical Oncology, 120:R108, 1994.

Other Reference Publication - OREF (91):
   Shaw et al., "Induction of apoptosis by wild-type **p53 in a human** colon tumor-derived cell line," Proc. Natl. Acad. Sci. USA, 89(10):4495-4499, 1992.

Other Reference Publication - OREF (98):
   Chen et al., "Genetic Mechanisms of Tumor Suppression by the **Human p53** Gene," Science, 250:1576-1580, 1990.

Other Reference Publication - OREF (99):
   Chen et al., "Expression of Wild-Type **p53 in Human** A673 Cells Suppresses Tumorigenicity but Not Growth Rate," Oncogene, 6:1799-1805, 1991.

Other Reference Publication - OREF (108):
   Takahashi et al., "Wild-Type but Not Mutant **p53 Suppresses the Growth of Human** Lung Cancer Cells Bearing Multiple Genetic Lesions," Cancer Research, 52:2340-2343, 1992.

Other Reference Publication - OREF (144):
   Fujiwara et al., "A Retroviral Wild-type **p53 Expression Vector Penetrates Human** Lung Cancer Spheroids and Inhibits Growth by Inducing Apoptosis," Cancer Research, 53:4129-4133, 1993.

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TITLE: Functional assay for transcriptional regulator genes

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APPL-NO: 08/ 447179

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PARENT-CASE:

   This is a continuation of application Ser. No. 08/046,033, filed Apr. 12,
1993, now abandoned which is a continuation-in-part of application Ser. No.
17/956,696 filed Oct. 1, 1992, abandoned.

US-CL-CURRENT: 435/6, 435/69.1 , 435/7.1 , 435/71.1 , 435/91.2

ABSTRACT:

   A method for determining whether an individual, i.e., a human, carries a
mutation in a gene which encodes a transcriptional regulator (e.g., a tumor
suppressor). The method includes the steps of obtaining a nucleic acid sample
comprising cDNAs encoding both alleles of the gene from a cell sample from the
individual; providing a nucleic acid construct comprising a binding site
operably linked to DNA encoding a detectable protein such that transcription of
the DNA encoding the detectable protein is regulated by interaction of the
transcriptional regulator polypeptide with the binding site; transfecting the
nucleic acid sample and the nucleic acid construct into cells with low levels
of the transcriptional regulator polypeptide; and then determining the levels
of the detectable protein in the transfected cells, this level being correlated
with the presence of a mutation in at least one of the alleles of the gene.

31 Claims, 7 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 4

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Detailed Description Text - DETX (8):

Two copies of the oligonucleotide GGAAT TCCTT GCCTT GCCTG GACTT GCCTG GCCTT GCCTT GGAAT TCC (SEQ ID NO:1) were cloned into the EcoRI restriction site of the pBSK (Stratagene, Inc.) plasmid to generate the plasmid pBSK(RGC). This oligonucleotide contains the region from nucleotide 103 to nucleotide 134 of fragment A isolated by Kern et al. (Science 252:1708). This **fragment A contains the p53** binding site located in the Ribosome Gene Cluster (RGC) (Kern et al., Science 252:1708; Farmer et al. Nature 358:83, 1992). A 3.6 kb HindIII-KpNI restriction fragment containing the murine for promoter (a 165 bp HindIII-XbaI restriction fragment from the plasmid pfosCAT.DELTA.56 (Gilman et al., Mol. Cell. Biol. 6:4305, 1986)), the open reading frame of the LacZ gene (a 3106 bp XbaI-BamHI restriction fragment), the small t intron and the polyadenylation signals of SV40, was then cloned into the plasmid pBSK SK(RGC) to generate the plasmid pRGCFos.DELTA.LacZ. The plasmid pFos.DELTA.LacZ differs from the plasmid pRGCFos.DELTA.LacZ by the absence of the RGC fragment.

Detailed Description Text - DETX (19):

In order to detect germline p53 mutations which inactivate the transcriptional activity of the wild-type protein, we designed a biological assay composed of 4 steps: (i) Amplification of p53 cDNA from normal cells, (ii) cloning of the p53 cDNA into a eukaryotic expression vector, (iii) transfection of the p53 vectors with a reporter plasmid for the transcriptional activity of p53, into a cell line which does not contain any p53 protein and (iv) analysis of the transfected cells. This assay requires a reporter plasmid which allows easy measurement of the transcriptional activity of p53. To this aim, we constructed the plasmid pRGCFos.DELTA.LacZ. This plasmid contains two copies of the RGC-**p53 binding fragment** in a head to head orientation. The wild-type **p53 protein has been shown to bind strongly to this DNA fragment** (Kern et al.,Science 252:1708) and this binding activates the transcriptional activity of recombinant promoters containing the RGC fragment (Farmer et al., supra). In contrast, the mutant proteins are unable to bind this sequence and to activate transcription (Farmer et al., supra). In the plasmid pRGCFos.DELTA.LacZ, the two RGC fragments are cloned upstream of a deletion mutant of the murine for promoter which has been shown to have no transcriptional activity (Gilman et al., supra). Cotransfection experiment using the pRGCFos.DELTA.LacZ plasmid and a wild-type p53 expression vector into Saos-2 cells results in approximately a 50 fold stimulation in the .beta.-galactosidase activity (Table 1). In contrast, transfection experiments with the plasmid pFos.DELTA.LacZ which does not contain the RGC fragment showed that the transcriptional activity of the for promoter is very weak in Saos-2 cells and that this activity is not inducible by the p53 protein (Table 1). Cotransfection experiments with the pRGCFos.DELTA.LacZ plasmid and vectors expressing mutant proteins at codon 143, 245, 248, 249, 258, 273, and 282, showed that these mutant p53 proteins were unable to activate the transcription of the RGC-for promoter. In contrast, cotransfection of the reporter plasmid with a vector expressing the mutant at codon 181 (a functionally silent mutation without biological significance (Frebourg et al., supra) revealed that the 181 mutation had not inactivated the transcriptional activity of the p53 protein (Table 1).

Detailed Description Text - DETX (28):

p53 was expressed in S. cerevisiae using P2, a vector containing the GAL1/10 promoter cloned into the Apal site and the CYC1 **terminat r** cloned into the SacII site of pRS314. pRS314 is a yeast low copy number replicating plasmid containing the TRP1 gene, CEN6 and ARSH4 (Sikorski and Hieter (1989) Genetics 122:19-27). p53 cDNAs containing various mutations were derived from bacterial expression plasmids constructed by Midgely et al. (Midgely et al. (1992) J Cell Sci 101:183-189). The **human p53 cDNAs as XbaI/HindIII fragments** into SpeI/NotI digested P2. The 5'-untranslated region in the p53 mRNA contains 55 nucleotides of the GAL1 mRNA followed by 5' CCCCGGATCC ACTAGAAATA ATTTTGTTTA ACTTTAAGAA GGAGATATAC GC-ATG-3' (SEQ ID NO:4) in the plasmids expressing the human 175H, 248W and 273H cDNAs. In plasmids expressing the human wild type and 285K cDNAs the underlined A is replaced by CCCCTCGA (SEQ ID NO:5). In the plasmids expressing murine p53 the underlined GC is replaced by AT.

Detailed Description Text - DETX (36):

The recipient strain for the gap repair experiments was initially transfected with a pLG.DELTA.178-derived lacZ reporter plasmid containing 3 copies of the p53 DNA-binding site adaptor oligonucleotide (in orientation-1, see above). 1 ng of the human wild type and 273H mutant p53 CDNA plasmid proSp53 (Matlashewski et al. (1987) Mol Cell Biol 7:961-963) and pR4.2 (Harlow et al. (1985) Mol Cell biol 5:1601-1610) were amplified for 30 cycles (92.degree. for 30", 60.degree. for 30", 72.degree. for 150") with 2 units AmpliTaq DNA polymerase in buffer containing 10 mM Tris pH 8.3, 50 mM KCI, 1.5 mM MgCl.sub.2, 0.1% Triton-x100, 200 .mu.M deoxynucleotides, 10% dimethyl sulfoxide, 100 .mu.M tetramethylammonium chloride and 5 .mu.g/ml primers, and the products were extracted with chloroform and precipitated with ethanol. The polymerase chain reaction (PCR) primers used were 5'-TGGATTGGCAGCCAGACTGCCTTCC-3' (SEQ ID NO:7) (11 nucleotides before the initiator methione codon) and 5'-GTGGGAGGCTGTCAGTGGGGAACAA-3' (SEQ ID NO:8) (14 nucleotides after the stop codon). The yeast expression plasmid containing the **human p53** 175H mutant (described above) was digested with StyI and the vector fragment (codons 160 to 347, see FIG. 3). The gapped plasmid and PCR products overlap by 477 base pairs at the 5'-end and 280 base pairs at the 3'-end. Approximately 100 ng of the gapped plasmid was mixed with .about.500 ng of PCR product, transfected by electroporation into the strain containing the lacZ reporter plasmid and 1/100th of the transfection mix was spread on plates containing 1M sorbitol, 2% glucose, 0.67% yeast nitrogen base (Difco), 1% casaminoacids (Difco) and 2.5% agar (Difco). To detect .beta.-galactosidase activity individual colonies were streaked onto plates containing 40 mg/1 X-gal (Sigma), 0.1M phosphate buffer pH 7.0, 2% galactose, 0.67% yeast nitrogen base (Difco), 1% casaminoacids (Difco) and 2.5% agar (Difco).

Detailed Description Text - DETX (39):

Truncation of this promoter at the XhoI site removes upstream activating sequences which normally control the activity of the CYC1 promoter (`minimal promoter`). The test promoter (`p53::CYC1 promoter`) contains a 33 base pair p53 DNA-binding sequence (Kern et al. supra) cloned into the XhoI site of the minimal promoter. The p53 expression plasmid contains a wild type **human p53** cDNA under the control of the GAL1 promoter:p53 expression is repressed by

glucose and stimulated by galactose. Table 2 shows that p53 stimulates lacZ expression, and that the effect requires both the presence of p53 protein (compare glucose with galactose) and the presence of the p53 DNA-binding site (compare the minimal promoter with the p53::CYC1 promoter). The effect of orientation and copy number of the 33 base pair sequence on wild type **human p53**-dependent transcription were analyzed (table 3).

Detailed Description Text - DETX (41):

   The ability of **human p53** mutants to stimulate transcription was tested with the reporter plasmid containing a single copy of the binding site oligo in the more effective orientation. Four mutants were examined (175H, 248W, 273H and 285K). Amino acids 175, 248 and 273 are hotspots for mutation in human tumors (Levine et al. (1991) Nature 351:453-456). Amino acid 248 is commonly mutated in the Li-Fraumeni hereditary cancer syndrome (Malkin et al. (1990) Science 250:1233-1238). The 175H mutant cooperates efficiently with ras in transformation assays and binds to hsc70, whereas the 273H mutant cooperates the inefficiently with ras and fails to bind to hsc70 (Hinds et al. (1990) Cell Growth and Differentiation 1:571-580). The 285K mutant was chosen because it binds well to the mutant p53-specific antibody PAb240 and fails to bind to the wild type p53-specific antibody PAb1620 in immunoprecipitation assays using mammalian cell extract (Bartek et al. (1990) Oncogene 5:893-899). Three of the mutants (175H, 248W and 273H) are completely inactive in transcription assays in yeast whereas the fourth mutant is weakly active (285K, table 4).

Detailed Description Text - DETX (43):

   The correlation between mutation and transcriptional inactivation suggests that it should be possible to detect p53 mutation using a simple yeast colony colour assay. Yeast will repair double stranded breaks in transfected plasmids by homologous recombination i.e, `gap repair` (Guthrie and Fink supra). If yeast are transfected with both a gapped plasmid and a suitable linear template they use the transfected template to repair the gap. It is thus possible to cotransfect yeast with a **fragment of p53** cDNA and a gapped p53 expression plasmid (FIG. 3), select for the auxotrophic marker on the plasmid and test colonies for .beta.-galactosidase activity on X-gal plates. To test this strategy, wild type and 273H mutant p53 CDNA plasmids (proSp53 and pR4.2) were amplified by the polymerase chain reaction using primers which span the open reading frame and cotransfected with a gapped expression plasmid into yeast containing a lacZ reporter plasmid. Non-homologous repair and integration events are expected to give rise to a small number of colonies in transfections with plasmid alone. Addition of the p53 PCR product produced a 75-fold increase in the number of colonies relative to that seen the gapped plasmid alone. 35 randomly picked colonies from the primary transfection plates were transferred to plates containing galactose and X-gal to induce p53 expression and assay .beta.-galactosidase activity. All colonies from the plate transfected with plasmid alone were white. One out of 35 colonies transfected with the 273H mutant was blue, and 30 of 35 colonies transfected with the wild type PCR product were blue.

Detailed Description Text - DETX (50):

   For one transformation, overnight culture of yeast cells were diluted to an

A.sub.600 of 0.2 in 10 ml of SDAcas liquid media, then grown to an A.sub.600 of
0.6-1.0 at 30.degree. C. The cells were pelleted by centrifugation, and then
washed in 1 ml LiOAc solution (0.1M lithium acetate, 10 mM Tris HCl, 1 mM EDTA
Na.sub.2, pH7.5). After centrifugation, cells were resuspended in 50 .mu.l of
LiOAc solution. These LiOAc treated cells were added to a 1.5 ml-tube
containing p53 expression vector with 50 .mu.g of carrier DNA, followed by the
addition of 300 .mu.l of LiOAc solution containing 40% of polyethylene glycol
3350-4000. This mixture was incubated at 30.degree. C. for 30 min with
shaking, and then at 42.degree. C. for 15 min. For the gap repair assays,
50-100 ng of the gapped vector and 50-200 ng of **p53 cDNA (a restriction
fragment** digested from a plasmid or PCR product) were introduced instead of the
p53 expression vector. The cells were collected, resuspended in 70-100 .mu.l
of sterile H.sub.2 O, and then spread onto a plate SC-leu. These conditions
generated between 100 and 3000 Leu.sup.+ colonies after incubation for 36-40
hours at 30.degree. C., depending on the transformation efficiency. Some of
the colonies (usually 50 colonies) were re-plated on SC -his -leu and further
incubated for 48 hours at 37.degree. C., and the number of colonies which grew
on the plate (His.sup.+, Leu.sup.+ phenotype) were counted.

Detailed Description Text - DETX (52):
   Messenger RNA was extracted from peripheral mononuclear cells, fibroblasts,
cell lines or frozen tumor tissue using a Micro-Fast Track mRNA Isolation Kit
Ver. 1.2 (Invitrogen). cDNA was synthesized from part of the RNA using
First-Strand cDNA Synthesis Kit (Pharmacia). To amplify p53 cDNA, 5 .mu.l of
first strand cDNA reaction was added to a PCR reaction. The p53 specific
primers used were as described in Example 1 (SEQ ID NOS: 1 and 2). PCR was
performed in 50 .mu.l of a solution containing 25 mM Tris-HCl pH 8.2, 17 mM
KCl, 6 mM (NH.sub.4).sub.2 SO.sub.4, 2.4 mM MgCl.sub.2, 180 .mu.M of each dNTP
and 2.5 units Pfu polymerase (Stratogene) using a Thermal Cycler (Cetus). To
compare the fidelity of reading, 2.5 units of Taq polymerase (Promega) was also
used instead of Pfu polymerase where indicated. Time and temperature were
programmed for 3 min at 95.degree. C., then 30-35 cycles consisting 1 min at
94.degree. C., 1 min at 58.degree. C., and 2 min at 72.degree. C., followed
by 5 min at 72.degree. C. After electrophoresis to evaluate the size of **p53
cDNA and the amount of the fragment,** the PCR product was introduced into yeast
directly or after phenol/chloroform extraction and ethanol precipitation
depending on the yield of DNA.

Detailed Description Text - DETX (55):
   Plasmids, gapped vector and **p53 cDNA fragment**

Detailed Description Text - DETX (57):
   The Ncol-Stul **fragment of p53** cDNA in pLS76(WT) were replaced by mutant
(175H, 248W and 273H) **human p53** cDNA(s) and called pLS76(175H), pLS76(248W) and
pLS76(273H), respectively, which were used as mutant p53 expression vectors.
For the gap repair assay, pSS16 was digested with HindIII and Stul, and then
the adhesive end of HindIII site was filled in by DNA polymerase (Klenow)
followed by dephosphorylation with calf intestinal alkaline phosphatase. After
fractionation by agarose gel electrophorecis, the fragment was purified by
phenol extraction, precipitated with ethanol, and then redissolved in an

appropriate volume of TE. The length of homology in the overlapped regions between p53 cDNA and this gapped vector are 201 and 173 bp at the 5' and 3' ends of p53 cDNA, respectively. A series of **human p53** expression vectors for mammalian cells were used for the source of p53 cDNA for the gap repair assay. Wild-type p53 cDNA was isolated from the plasmid pC53-SN3 (Baker et al., 1990 supra). p53 cDNA which encodes mutant proteins at codon 143A, 245C, 248W, 249S, 252P, 258K and 273H were derived from the **human p53** expression vectors described above (Example 1). p53 cDNA which encodes mutant proteins at codon 156P, 175H, 248Q and 281E were derived from the pJ7.OMEGA. vectors containing each mutant p53 cDNA.

Detailed Description Text - DETX (61):
    This example describes a method which is based on the ability to separate p53 alleles from a patient's p53 cDNA pool using homologous recombination and a gapped p53 excision repair vector. Because homologous recombination is a rare event, it was necessary to set up a method by which to select only for yeast that have successfully accomplished homologous recombination and therefore incorporated a patient's p53 allele into the expression vector. This was achieved using a pSS16 gap excision repair vector (FIG. 4). In its intact circular form, the yeast autonomously replicating sequence (ARS) element allows replication of the LEU2 selectable marker. Since LEU2 is separated from the ARS by the ADH1 promoter driving expression of the **p53 cDNA, when a portion of p53** is excised, LEU2 replication will not occur until homologous recombination repairs the gapped sequence. Therefore, when the transformants are plated on SC -leu, it is possible to select for the rare yeast in which the p53 cDNA repairs the gapped sequence by homologous recombination yielding an intact vector (indicated as pLS76 in FIG. 4), when the transformants are plated on SC-leu media. The pSS16 gapped vector also includes a centromere sequence (CEN) to allow the repaired vector, pLS76, to segregate accurately with a single copy during cell division thus producing yeast which contain only a single allele of p53 per cell.

Detailed Description Text - DETX (67):
    Because germline p53 mutations have been found between codon 71 to 325, the expression vector pSS16 was designed to make the gapped region from codon 67 to 347 (FIG. 5). The efficiency of homologous recombination between this new gapped vector and the p53 cDNA was then tested by determining both the number of colonies that grew on SC-leu plates and the difference in the number of colonies which were able to grow on the SC-his-leu plates for wild-type and mutants p53. When wild-type or mutant **p53 cDNA fragments** were introduced into the yeast with this gapped vector (p.SS16 HindIII-StuI digest), 2.times.10.sup.2 -1.4.times.10.sup.3 colonies were observed on SC-leu plates. In contrast, only 6 colonies were detected in transfection with the gapped vector alone. When the p53 cDNA that was introduced was wild-type, 99.3% (149 of 150) also demonstrated a His.sup.+ phenotype. In contrast, when mutant p53 cDNAs (143A, 156P, 175H, 245C, 248W, 248Q, 252P, 258K, 273H, 281E and 307stop) were introduced, no His.sup.+ colonies were observed (0/50 for each mutant clone). Representative results are shown in FIG. 5. These results indicate that the gapped vector is successfully repaired by homologous recombination with p53 CDNA, and demonstrate that wild-type p53 cDNA can be discriminated from mutant p53 cDNAs by this selectable transactivation system.

Detailed Description Paragraph Table - DETL (2):
TABLE 2 _____ .beta.-galactosidase
assays. Expression plasmid lacZ reporter plasmid Glucose Galactose
_____ No insert Intact CYC1 promoter 180 .+-.
14 546 .+-. 23 Wild type **human p53** Intact CYC1 promoter 205 .+-. 27 523
.+-. 20 No insert Minimal promoter 1 .+-. 1 1 .+-. 1 Wild type **human p53**
Minimal promoter 1 .+-. 1 2 .+-. 2 No insert p53 CYC1 promoter 1 .+-. 1 3
.+-. 1 Wild type **human p53** p53 CYC1 promoter 1 .+-. 1 139 .+-. 1
_____ Units of .beta.-galactosidase were
calculated as described in the methods section and represent the mean of three
independent assays .+-. standard deviation. The intact CYC1 promoter is mildly
repressed by glucose (Guarente and Ptashne (1981) Proc Natl Acad Sci USA 78:
2199-2203).

Detailed Description Paragraph Table - DETL (4):
TABLE 4 _____ .beta.-galactosidase assays
using different p53 mutants. **Human p53** gene Minimal promoter p53 CYC1
promoter _____ wild type 3 .+-. 1 334 .+-. 13
175H 2 .+-. 2 3 .+-. 1 248W 3 .+-. 1 2 .+-. 1 273H 3 .+-. 1 3 .+-. 1 285K 3
.+-. 1 24 .+-. 4 _____ Cells were grown in
medium containing galactose.

Detailed Description Paragraph Table - DETL (6):
TABLE 6 _____ Cotransformation of yeast
with gapped vector and PCR product of p53 cDNA. Input DNA p53 cDNA (DNA
polymerase, His.sup.+ phenotype.sup.b gapped vector.sup.a each dNTP (.mu.M)
% (No. of colonies) _____ - -- NA.sup.c -
non-PCR.sup.d NA + -- 0 (0/3).sup.e + non-PCR 100 (100/100) + PCR (Pfu, 50
.mu.M) 100 (100/100) + PCR (Pfu, 100 .mu.M) 100 (100/100) + PCR (Taq, 50
.mu.M) 86 (43/50) + PCR (Taq, 200 .mu.M) 76 (114/150) + PCR (Taq, 600
.mu.M) 42 (26/50) _____ .sup.a plasmid pSS16
HindIIIStuI digest (50 ng) .sup.b The part of individual transformants were
assayed for His.sup.+ phenotype. .sup.c not applicable. .sup.d wildtype **p53
cDNA fragment** (100 ng) deribed from pC53SN3 .sup.e only 3 colonies on SC leu
plate

Other Reference Publication - OREF (11):
   Dalbagni et al., Proceedings of the AACR 33:373, Abstract .pi.2229 (1992),
"P53 and chromosome 17 abnormalities in human bladder tumors".

Other Reference Publication - OREF (17):
   Funk et al., Molecular and Cellular Biology 12:2866-2871 (1992), "A
Transcriptionally Active DNA-Binding Site for **Human p53** Protein Complexes".

Other Reference Publication - OREF (18):

Hsu et al., Nature 350:427-428 (1991), "Mutational hotspot in the **p53 gene in human** hepatocellular carcinomas".

Other Reference Publication - OREF (30):
Romano et al., Oncogene 4:1483-1488 (1989), "Identification and characterization of a **p53 gene mutation in a human** osteosarcoma cell line".

US-PAT-NO: 5736338

DOCUMENT-IDENTIFIER: US 5736338 A

TITLE: Method of diagnosing Neoplastic disease by detecting
                increased expression of human MDM2 protein

DATE-ISSUED: April 7, 1998

INVENTOR-INFORMATION:
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APPL-NO: 08/ 390517

DATE FILED: February 17, 1995

PARENT-CASE:

  This application is a division of application Ser. No. 08/044,619 U.S.
Pat. No. 5,420,263 filed Apr. 7, 1993, which is a continuation-in-part of
Ser. No. 07/903,103 U.S. Pat. No. 5,411,860 filed Jun. 23, 1992, which is a
continuation-in-part of Ser. No. 07/867,840 (abandoned) filed Apr. 7, 1992 .

US-CL-CURRENT: 435/7.1, 435/7.23 , 435/7.92 , 530/387.7 , 530/388.85

ABSTRACT:

  In certain human tumor cells, the gene encoding MDM2 protein is amplified
and expression of MDM2 protein is elevated. Since human MDM2 protein binds to
human p53, excess MDM2 protein apparently releases a cell from p53-regulated
growth. Detection of elevated amounts of human MDM2 protein thus can be used
to diagnose neoplastic disease in a human.

11 Claims, 24 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 17

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Abstract Text - ABTX (1):
  In certain human tumor cells, the gene encoding MDM2 protein is amplified
and expression of MDM2 protein is elevated. Since human MDM2 protein binds to

human p53, excess MDM2 protein apparently releases a cell from p53-regulated growth. Detection of elevated amounts of human MDM2 protein thus can be used to diagnose neoplastic disease in a human.

Brief Summary Text - BSTX (6):
  While there exists an enormous body of evidence linking **p53 gene mutations to human** tumorigenesis (Hollstein et al., 1991, Science 253: 49-53) little is known about cellular regulators and mediators of p53 function.

Brief Summary Text - BSTX (7):
  Hinds et al. (Cell Growth & Differentiation, 1: 571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated was oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253: 49-53. The transformed fibroblasts were found to produce elevated levels of **human p53** protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Brief Summary Text - BSTX (8):
  Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type **human p53** gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4: 81-96.

Brief Summary Text - BSTX (9):
  Hinds et al., supra also expressed **human p53** protein in transformed rat cells. When the expressed **human p53** was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified $M.sub.r$ 90,000 protein was coimmunoprecipitated. This suggested that the rat $M.sub.r$ 90,000 protein is in a complex with the **human p53** protein in the transformed rat cell line.

Brief Summary Text - BSTX (19):
  Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (22):
  Still another object of the invention is to provide polypeptides which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (23):

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to **human p53** in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

Drawing Description Text - DRTX (11):

FIG. 6C. Random **fragments of p53** were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Detailed Description Text - DETX (13):

It has been found that amino acid residues 13-41 of **p53** (See SEQ ID NO: 1) are necessary for the interaction of MDM-2 and **p53**. However, additional residues on either the amino or carboxy **terminal** side of the **peptide** appear also to be required. Nine to 13 additional **p53** residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from **p53**-regulated growth control in sarcomas, the use of **p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53**-regulated growth control.

Detailed Description Text - DETX (14):

Suitable **p53-derived peptides** for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

Detailed Description Text - DETX (15):

DNA encoding the MDM2-binding, **p53-derived peptide,** or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus, or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, inter alia encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of **peptide, the goal is to alleviate the sequestration of p53** by MDM2.

Detailed Description Text - DETX (18):

The human MDM2 gene has now been identified and cloned. Recombinant derived

hMDM2 has been shown to bind to **human p53**. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

Detailed Description Text - DETX (27):

   To determine whether the hMDM2 protein could bind to **human p53** protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in FIG. 1 from nucleotide 312 to 2176. A 42 bp black bettie virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15: 3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251: 1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Detailed Description Text - DETX (33):

   The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (FIG. 2, lanes 2 and 3). However, when in vitro translated **human p53** was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association in vitro (FIG. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, Science 251: 1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (FIG. 2, lanes 8 and 9). When an in vitro translated mutant form of p53 (175.sup.his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

Detailed Description Text - DETX (59):

   This assay was then applied to mapping the interaction domains of each protein. Full length cDNA **fragments encoding MDM2 or p53** were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

Detailed Description Text - DETX (60):

   METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K. W., et al., Nucl. Acids Res. 17: 3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000

pb, cloned into lexA(1-202)+PL (Ruden, D. M., et al., Nature 350: 250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive .beta.-galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent clones are diagrammed in FIG. 6A. The MDM2 sequences of the remaining 15 **p53-dependent clones coded for peptides** ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of FIG. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Detailed Description Text - DETX (61):

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein **C-terminal** to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three **p53** sequences shown in FIG. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated **fragments**.

Detailed Description Text - DETX (62):

The resultant yeast colonies were examined for .beta.-galactosidase activity in situ. Of approximately 5000 clones containing MDM2 **fragments** fused to the lexA DNA binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of .beta.-galactosidase (about 5-fold less than the other fifteen clones) and .beta.-galactosidase expression was independent of **p53** expression (FIG. 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2-protein fused to lexA had no measurable .beta.-galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino **terminal** region of MDM2 (FIG. 6B). The .beta.-galactosidase activity of these clones was dependent on **p53** co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length **p53** contained MDM2 codons 1

to 118 (FIG. 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated **fragments** of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

Detailed Description Text - DETX (63):

In a converse set of experiments, yeast clones containing **fragments of p53** fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (FIG. 6C). The minimal overlap between these three **fragments** contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the **fragments** required 9-12 amino acids on either side of codons 13-41 for activity (FIG. 6C). To further test the idea that the amino **terminal** region of **p53** was required for MDM2 binding, we generated an additional yeast strain expressing the lexA-DNA binding domain fused to **p53** codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-**terminus of p53** were required for the interaction.

Detailed Description Text - DETX (64):

Sequence analysis showed that all **p53 and MDM2 fragments** noted in FIG. 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in FIG. 6 expressed the relevant proteins at similar levels, as shown by Western blotting (FIG. 7).

Other Reference Publication - OREF (3):

Hinds, et al., "Mutant **p53 DNA Clones From Human** Colon Carcinoma Cooperate With Ras in Transforming Primary Rat Cells: A Comparison of the Hot Spot Mutant Phenotypes", Cell Growth & Differentiation, 1:561-580 (1990).

Other Reference Publication - OREF (6):

Oliner, et al., "Amplification of a Gene Encoding a **p53-Associated Protein in Human** Sarcomas", Nature, 358:80-83 (1992).

Other Reference Publication - OREF (8):

Leach, et al., "**p53 Mutation and MDMS Amplification in Human** Soft Tissue Sarcomas", Cancer Research 53:2231-2234 (1993).

US-PAT-NO: 5726024

DOCUMENT-IDENTIFIER: US 5726024 A

TITLE: p53as protein and antibody therefor

DATE-ISSUED: March 10, 1998

INVENTOR-INFORMATION:
NAME   CITY   STATE   ZIP CODE COUNTRY
Kulesz-Martin; Molly F.   Buffalo   NY   N/A   N/A

APPL-NO: 08/ 644291

DATE FILED: May 10, 1996

PARENT-CASE:

   This is a Continuation-in-part of U.S. patent application Ser. No.
08/259,612 filed Jun. 14, 1994 which is a Continuation-in-part of U.S. patent
application Ser. No. 08/195,952 filed Feb. 11, 1994, now abandoned, which is
a Continuation-in-part of U.S. application Ser. No. 08/100,496, filed Aug.
2, 1993.

US-CL-CURRENT: 435/7.1, 435/7.23 , 530/387.7 , 530/388.8 , 530/388.85
          , 530/389.7

ABSTRACT:

   The invention comprises plasmids and viral vectors containing an animal
p53as cDNA sequence. A portion of the p53as sequence may be identified to a
position of wild type p53 gene from the same animal. In preferred embodiments,
the p53as is mouse or human p53as. A preferred viral vector is baculovirus
vector. The invention further includes antibodies both polyclonal and
monoclonal, to p53as and to at least a **portion of human p53** intron 10 sequence
encoding SLRPFKALVREKGHRPSSHSC (SEQ ID NO: 1) which is related to p53as
sequences and plasmids and viral vectors containing such sequences. All of the
above find utility in studying p53 and p53as and their relative expressions
which is believed important for detection and control of malignant cells and
their susceptibility to treatment agents. The antibodies can detect the
presence of p53as and related sequences and when injected into cells could
cause cell cycle arrest and the plasmids and viral vectors, with appropriate
promotors, can cause expression of the p53as and p53 intron 10 sequences which
can affect cell growth and perhaps arrest certain malignancies.

5 Claims, 21 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 13

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Abstract Text - ABTX (1):
   The invention comprises plasmids and viral vectors containing an animal
p53as cDNA sequence. A portion of the p53as sequence may be identified to a
position of wild type p53 gene from the same animal. In preferred embodiments,
the p53as is mouse or human p53as. A preferred viral vector is baculovirus
vector. The invention further includes antibodies both polyclonal and
monoclonal, to p53as and to at least a **portion of human p53** intron 10 sequence
encoding SLRPFKALVREKGHRPSSHSC (SEQ ID NO: 1) which is related to p53as
sequences and plasmids and viral vectors containing such sequences. All of the
above find utility in studying p53 and p53as and their relative expressions
which is believed important for detection and control of malignant cells and
their susceptibility to treatment agents. The antibodies can detect the
presence of p53as and related sequences and when injected into cells could
cause cell cycle arrest and the plasmids and viral vectors, with appropriate
promotors, can cause expression of the p53as and p53 intron 10 sequences which
can affect cell growth and perhaps arrest certain malignancies.

Brief Summary Text - BSTX (5):
   This application provides further support for the utility of p53as
expression from plasmids and vectors such as those described in application
Ser. No. 08/195,952. Further evidence is presented that p53as protein has
"tumor suppressor" activity in mouse and human cells, activates transcription
through **p53 target sequences of mouse and human** cells, and forms tetramers, a
DNA binding form observed for the tumor suppressor gene p53as. This
Application further claims the use of antibodies to human p53as for diagnosis
of human cancers, the use of antibodies to human p53as for determination of
prognosis of human cancers and use of antibodies to human p53as for determining
a treatment plan for individual patient cancers.

Brief Summary Text - BSTX (11):
   As described in the parent applications, the invention also comprises
plasmids and viral vectors containing a p53as cDNA sequence. A preferred viral
vector is baculovirus vector. The invention further includes antibodies both
polyclonal and monoclonal, to p53as and to at least a **portion of human p53**
intron 10 sequence encoding SLRPFKALVREKGHRPSSHSC which is related to p53as
sequences and plasmids and viral vectors containing such sequences.

Drawing Description Text - DRTX (17):
   FIGS. 10A, 10B and 10C show colony formation of Saos-2 cells transfected
with CMV plasmids. FIG. 10A shows a control without a cDNA insect. FIG. 10B
shows a p53as cDNA insect and FIG. 10C shows a p53 cDNA insect. Plasmid
containing p53as containing a CMV promoter (which drives expression of p53as in
the target mammalian cell) is introduced into **human   steosarcoma cells which
lack p53** which stops tumor cell growth and reduces the number and area of
colonies present in the culture dishes. This assay is commonly used to show

tumor suppressor activity of various genes and p53 used as a control shows
similar activity, thus wild type p53as acts as a tumor suppressor. In
contrast, a plasmid containing an alternatively spliced mutant p53 sequence had
transforming activity (Eliyahu et al., Oncogene 3,313-321, 1988). Tumor
suppressor activity was found in human cells as well as mouse cells. Human
osteosarcoma cell line Saos-2 was transfected using lipofectin, BRL0 with 5 ng
of the pCMV plasmids containing the p53 or p53as cDNA indicated and a neomycin
resistance gene. Vector without the cDNA insert was used as a control.
Forty-eight hours after transfection, cells were trypsinized, passaged at a 1:4
ratio and cultured in media containing 500 .mu.g/ml G418 for 3 weeks. Colonies
were fixed in methanol and stained with Giemsa and measured using an image
analysis system (Spectra Services).

Detailed Description Text - DETX (4):
    Plasmids containing the cDNA sequence unique to p53as are included in this
invention. One such plasmid is pBSp53as which contains full length
alternatively spliced **p53** cDNA. pBSp53as was constructed from **p53** cDNA
beginning at nt -111 of the (where 1 is the first ATG encoding methionine) and
ending at nt 1539, cloned into the EcoRI and BamHI sites of pBluescript SK
under the control of a T3 phage promoter. The N**-terminal fragment of wt p53**
was amplified by reverse transcriptase/polymerase chain reaction (RT-PCR) from
a mouse epidermal cell RNA template and the C**-terminal fragment** of p53as was
amplified by PCR from plasmid p6.4 (which contains an alternatively spliced **p53**
cDNA; Han and Kulesz-Martin, Nucl. Acids Res. 20: 1979-81, 1992) using
primers which contained a StuI restriction site at the 5' end of the sense
primer (AGTCAGGCCTTAGAGTTAAAGGATGCCCATGCTACAGA) (SEQ ID NO:2) and a
BamHI site
at the 5' end of the antisense primer. pBSp53 was made from pBSp53as by
replacement of the StuI/BamHI C**-terminal fragment** of p53as cDNA with the
StuI/BamHI segment of wild type **p53** cDNA from plasmid pLSVNc51 (ref. Oren). In
particular, cDNA for the N**-terminus of p53** (nt -111 to 1090) was made using
template RNA from 291 nontransformed epidermal cells by means of a reverse
transcriptase reaction, amplified by PCR and cloned into the EcoRI and BamHI
sites of pBluescript SK under the control of a T3 phage promoter to create
plasmid pBSRS 13. The primers used for PCR were: sense,
AGTCGAATTCATTGGGACCATCCTGGCT (SEQ ID NO:3), antisense,
AGTCGGATCCTGGAGTGAGTGAGCCCTGCTGTCT (SEQ ID NO: 4). These primers
contained an
EcoRI restriction site at the 5' end of the sense primer and a BamHI site at
the 5' end of the antisense primer (denoted by underlining). The C**-terminal**
p53 cDNA (nt1028 to 1539) was amplified by PCR from plasmid p6.4 (which
contains an alternatively spliced **p53** cDNA) using primers which contained a
StuI restriction site at the 5' end of the sense primer
(AGTCAGGCCTTAGAGTTAAAGGATGCCCATGCTACAGA) (SEQ ID NO:2) and a BamHI site
at the
5' end of the antisense primer (as in Hart and Kulesz-Martin, Nucl. Acids Res.
20: 1979-81, 1992). The StuI to BamHI segment of this PCR reaction product was
then ligated to the StuI and BamHI sites of plasmid pBSRS13 to create plasmid
pBSp53as, containing a full length alternatively spliced **p53** cDNA. To
construct pBSp53, the StuI and BamHI **fragment from wt p53 cDNA was substituted**
f r the StuI and BamHI fragment of the p53as cDNA in pBSp53as.

Detailed Description Text - DETX (16):

In contrast to cotranslation of p53 and p53as proteins, mixture of lysates
containing each protein translated individually did not show inactivation of
p53as by p53 protein for DNA binding, and PAb421 supershifted only one band,
suggesting that p53 and p53as must be translated together for association to
occur (FIG. 6). This is consistent with the report that oligomerization
between **human p53** and mouse p53 occurs when they are cotranslated, but not when
mixed (Milner and Medcalf, 1991).

Detailed Description Text - DETX (25):

Methods were per manufacturers instructions (Invitrogen) and materials and
included linear AcMNPV DNA and transfer vector (e.g. pVL 1392, pVL 1393) and
insect cell line S. frugiperda Sf9, propagated at 27.degree. C. in Grace's
supplemented insect medium containing 10% fetal bovine serum (GIBCO) and 10
.mu.g/ml gentamycin sulfate. pVL1393BGB53 baculovirus vector containing wt **p53
cDNA was constructed by inserting the Bg1II/BamHI fragment of pLSVNc51
(including the entire wt p53** cDNA) into the pVL 1393 vector. pVL1393Asp
baculovirus vector (containing p53as cDNA) was constructed by replacement of
the StuI/BamHI C-**terminal fragment** of p53as cDNA (Han and Kulesz-Martin, Nucl.
Acids Res. 1992) with the BamHI/StuI **fragment** of the pVL1393BGB53 vector (see
above). To purify recombinant viruses, Sf9 cells were cotransfected with
linear AcMNPV DNA and the transfer vector containing p53as and grown for 3
days. Recombinant viruses were identified by plaque assays or serial
dilutions. Alternatively, **p53** and p53as baculovirus contructions will be
cotransfected with linearized (PharMingen) virus DNA which allows propagation
of only recombinant virus. Virus stocks which resulted in **p53** as expression in
insect cells (assayed by immunoblotting using anti-p53as antibody) were
expanded and used to infect insect cells for the flow cytometry studies.

Detailed Description Text - DETX (42):

Because **human and mouse p53** proteins form complexes in cells, the construct
containing mouse p53as cDNA is claimed for the purposes of gene expression in
mammalian cells and nonmammalian cells for research purposes, including human
cells, and for gene therapy in humans. In addition, a purified plasmid
construct containing the human p53as homologue of mouse p53as, defined by
insertion of human intron 10 sequences into a sequence containing wt p53 DNA
(as defined in original patent application Ser. No. 08/100,486) is claimed for
research purposes in mammalian and nonmammalian cells, and for gene therapy in
humans.

Detailed Description Text - DETX (43):

Table 1. shows reactivities of antibodies against p53 proteins. Mouse **p53
has 390 amino acids; human p53** 393 amino acids. All antibodies are mouse
monoclonals commercially available from Oncogene Science, Cambridge Mass.,
except ApAs rabbit polyclonal specific for p53as protein which was made in Dr.
Kulesz-Martin's laboratory, RPCI. Sources: Oncogene Science Catalogue, p. 8,
1992; Vajtesek et al., J. of Immunolog. Methods 151:237-244, 1992, .sup.a

Wade-Evans, A. and Jenkins, J. R. EMBO J., 4:699-706, 1985, .sup.b Gannon, EMBO, 9:1595-1602, 1990, .sup.c Stephen, C. W. and Lane, D. P., J. Mol. Biol., 5:577-583, 1992 and .sup.d Kulesz-Martin et al., Mol. Cell. Biol., in press, March 1994.

Detailed Description Text - DETX (47):

Tables 3 and 4 demonstrate that p53as has transcriptional activity. Plasmids containing p53as, or **p53 as a control, were introduced into mouse or human** cells along with a plasmid containing a p53 binding site upstream of a reporter gene. The reporter was activated by p53as, with p53 as a positive control, but not by the vector without p53as. By comparison, the background activity of the reporter plasmid alone was low. The reporter sequences 50-2 and PC13 are a promoter-enhancer sequence (Zambetti et al., Genes Dev. 6, 1143-1152, 1992) and a consensus p53 binding sequence (Kern et al., Science 256, 827-830, 1992). p53 as was active on mouse (50-2) or **human (PC13) p53** binding sequences, and in both mouse and human cells.

Detailed Description Text - DETX (51):

For example, minor cells which express different ratios of p53as and p53, as detected at the RNA level, or at the protein level by anti-p53as antibodies may have different characteristics or different sensitivity to anti-cancer treatments. Different characteristics identifiable by reactivity with p53as antibodies could aid in the diagnosis of cancer, proguosis for individual patients with tumors and decisions about treatment based on the competency of the p53as functions in the tumor. Antibodies to **p53 are being used clinically in diagnosis of human cancers because expression of p53** at detectable levels has been found to correlate with cancer prognosis for a variety of human tumor types. It is known that tumors with defective **p53 genes (more than 50% of all human** cancers) fail to control cell cycle progesssion normally. Since p53 and p53as have different cell cycle associations, tumor typing using specific p53as antibodies and specific anti-p53 antibodies may increase the value of typing individual tumors according to their expression of tumor suppressor gene products such as p53. Such tumor typing may provide useful information in the diagnosis, prognosis, and treatment strategy of individual patient cancers.

Detailed Description Paragraph Table - DETL (1):
TABLE 1

Reactivites of Antibodies Agains p53 Proteins wt wt cell frozen paraffin Ab Species conform denatured mutated p53 p53as epitope IP WIB staining sections sections

PAb421.sup.a mu/hu + + + + - 370-378 + +/- + + - PAb246.sup.a mu + - - + + 88-109 + - + + nt PAb240.sup.bc mu/hu - + + + (-) 210-214 + + + + - ApAs.sup.d mu + + nt - + (364-381) + + + nt nt

Mouse **p53 has 390 amino acids; human p53** 393 amino acids. All antibodies are mouse monoclonals commercially available from Oncogene Science, Cambridge MA, except ApAs rabbit polyclonal specific for p53as protein which was made in Dr. KuleszMartin's laboratory, RPCI. Sources: Oncogene Science Catalogue, p. 8,

1992; Vajtsesek et al., J. of Immunolog. Methods 151:237-244, 1992, .sup.a
WadeEvans, A. and Jenkins, J. R. EMBO J., 4:699-706, 1985, .sup.b Gannon,
EMBO, 9:1595-1602, 1990, .sup.c Stephen, C. W. and Lane, D. P., J. Mol. Biol.
5:577-583, 1992 and .sup.d KuleszMartin et al., Mol. Cell. Biol., in press,
Man 1994.

Claims Text - CLTX (2):
   a) reacting the cell sample with an antibody which specifically binds to
mammalian p53as protein and does not bind to normal **p53 from the same species
wherein said antibody binds to an epitope present in a peptide** unique to p53as,
said **peptide** occurring within the final 50 carboxyl **terminal** amino acids of
p53as;

Claims Text - CLTX (6):
   3. The method of claim 1 wherein the normal **p53 protein is a human p53**
protein.

Other Reference Publication - OREF (5):
   Bargonetti, J. et al. (1993) A Proteolytic **Fragment From the Central Region
of p53** Has Marked Sequence-Specific DNA-Binding Activity When Generated from
Wild-Type but not from Oncogenic Mutant p53 Protein, Genes & Development, 7,
pp. 2565-2574.

Other Reference Publication - OREF (6):
   Bischoff, J. et al., (1992) **Human p53** Inhibits Growth in Schizosaccharomyces
pombe, Molecular and Cellular Biology, vol. 12, No. 4, pp. 1405-1411.

Other Reference Publication - OREF (13):
   Funk, W. et al., (1992) A Transcriptionally Active DNA-Binding Site for
Human p53 Protein Complexes, Mol. and Cell. Biol., pp. 2866-2871.

Other Reference Publication - OREF (32):
   Nigro, J. et al., (1992) **Human p53** and CDC2Hs Genese Combine to Inhibit the
Proliferation of Saccharomyces cerevisiae, Molecular and Cellular Biology, pp.
1357-1365.

Other Reference Publication - OREF (33):
   Oliner, J. et al., (1992) Amplification of a Gene Encoding a **p53-Associated
Protein in Human** Sarcomas, Nature, vol. 358, pp. 80-83.

Other Reference Publication - OREF (40):
   Vojtesek, B. et al. (1992) An Immunochemical Analysis of the **Human Nuclear
Phosphoprotein p53,** Journal of Immunological Methods, 151, pp. 237-244.

US-PAT-NO: 5708136

DOCUMENT-IDENTIFIER: US 5708136 A

TITLE: Polypetides which bind to human MDM2

DATE-ISSUED: January 13, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Burrell; Marilee	Cambridge	MA	N/A	N/A
Hill; David E.	Arlington	MA	N/A	N/A
Kinzler; Kenneth W.	Baltimore	MD	N/A	N/A
Vogelstein; Bert	Baltimore	MD	N/A	N/A

APPL-NO: 08/ 390516

DATE FILED: February 17, 1995

PARENT-CASE:

   This application is a divisional application of Ser. No. 08/044,619, filed
Apr. 7, 1993, now U.S. Pat. No. 5,420,263, which is a continuation-in-part
of Ser. No. 07/903103, filed Jun. 23, 1992, now U.S. Pat. No. 5,411,860,
which is a continuation-in-part of Ser. No. 07/867,840, filed Apr. 7, 1992,
now abandoned.

US-CL-CURRENT: 530/324, 530/300

ABSTRACT:

   A human gene has been discovered which is genetically altered in human tumor
cells. The genetic alteration is gene amplification and leads to a
corresponding increase in gene products. Detecting that the gene, designated
hMDM2, has become amplified or detecting increased expression of gene products
is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and
allows the cell to escape from p53-regulated growth.

13 Claims, 24 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 18

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Abstract Text - ABTX (1):
   A human gene has been discovered which is genetically altered in human tumor

cells. The genetic alteration is gene amplification and leads to a
corresponding increase in gene products. Detecting that the gene, designated
hMDM2, has become amplified or detecting increased expression of gene products
is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and
allows the cell to escape from p53-regulated growth.

Brief Summary Text - BSTX (6):
   While there exists an enormous body of evidence linking **p53 gene mutations
to human** tumorigenesis (Hollstein et al., 1991, Science 253:49-53) little is
known about cellular regulators and mediators of p53 function.

Brief Summary Text - BSTX (7):
   Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that
p53 cDNA clones, containing a point mutation at amino acid residue 143, 175,
273 or 281, cooperated with the activated ras oncogene to transform primary rat
embryo fibroblasts in culture. These mutant p53 genes are representative of
the majority of mutations found in human cancer. Hollstein et al., 1991,
Science 253:49-53. The transformed fibroblasts were found to produce elevated
levels of **human p53** protein having extended half-lives (1.5 to 7 hours) as
compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Brief Summary Text - BSTX (8):
   Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric
protein complex with the cellular heat shock protein hsc70. While residue 273
or 281 mutants do not detectably bind hsc70, and are poorer at producing
transformed foci than the 175 mutant, complex formation between mutant p53 and
hsc70 is not required for p53-mediated transformation. Complex formation does,
however, appear to facilitate this function. All cell lines transformed with
the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the
wild-type **human p53** gene does not possess transforming activity in cooperation
with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

Brief Summary Text - BSTX (9):
   Hinds et al., supra also expressed **human p53** protein in transformed rat
cells. When the expressed **human p53** was immunoprecipitated with two p53
specific antibodies directed against distinct epitopes of p53, an unidentified
$M.sub.r$ 90,000 protein was coimmunoprecipitated. This suggested that the rat
$M.sub.r$ 90,000 protein is in a complex with the **human p53** protein in the
transformed rat cell line.

Brief Summary Text - BSTX (19):
   Yet another object of the invention is to provide methods for identifying
compounds which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (22):
   Still another object of the invention is to provide polypeptides which

interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (23):

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to **human p53** in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

Brief Summary Text - BSTX (30):

FIGS. 6A-C show the determination of MDM2 and p53 domains of interaction. FIG. 5A and FIG. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive .beta.-galactosidase reporter). Yeast clones expressing .beta.-galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined. .beta.-galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. FIG. 6C. Random **fragments of p53** were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Brief Summary Text - BSTX (45):

It has been found that amino acid residues 13-41 of **p53** (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and **p53**. However, additional residues on either the amino or carboxy **terminal** side of the **peptide** appear also to be required. Nine to 13 additional **p53** residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from **p53**-regulated growth control in sarcomas, the use of **p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53**-regulated growth control.

Brief Summary Text - BSTX (46):

Suitable **p53-derived peptides** for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

Brief Summary Text - BSTX (47):

DNA encoding the MDM2-binding, **p53-derived peptide,** or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a

retrovirus, DNA virus, or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, inter alia encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of **peptide, the goal is t alleviate the sequestration of p53** by MDM2.

Brief Summary Text - BSTX (50):

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to **human p53**. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

Detailed Description Text - DETX (8):

To determine whether the hMDM2 protein could bind to **human p53** protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in FIG. 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Detailed Description Text - DETX (14):

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (FIG. 2, lanes 2 and 3). However, when in vitro translated **human p53** was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association in vitro (FIG. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (FIG. 2, lanes 8 and 9). When an in vitro translated mutant form of p53 (175.sup.his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

Detailed Description Text - DETX (41):

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA **fragments encoding MDM2 or p53** were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

Detailed Description Text - DETX (43):

Full length MDM2 cDNA in pBluescript SK+ (Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K. W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D. M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive .beta.-gaiactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galaclose (p53 induction); all tested positive in the presence of galaclose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent clones are diagrammed in FIG. 6A. The MDM2 sequences. of the remaining 15 **p53-dependent clones coded for peptides** ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of FIG. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Detailed Description Text - DETX (44):

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-**terminal** to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three **p53** sequences shown in FIG. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated **fragments**.

Detailed Description Text - DETX (45):

The resultant yeast colonies were examined for .beta.-galactosidase activity in situ. Of approximately 5000 clones containing MDM2 **fragments** fused to the lexA DNA binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of .beta.-galactosidase (about 5-fold less than the other fifteen clones) and/ .beta.-galactosidase expression was independent of **p53** expression (FIG. 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This

acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable .beta.-galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino **terminal** region of MDM2 (FIG. 6B). The .beta.-galactosidase activity of these clones was dependent on **p53** co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length **p53** contained MDM2 codons 1 to 118 (FIG. 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated **fragments** of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

Detailed Description Text - DETX (46):

In a converse set of experiments, yeast clones containing **fragments of p53** fused to the 1542 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (FIG. 2C). The minimal overlap between these three **fragments** contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the **fragments** required 9-12 amino acids on either side of codons 13-41 for activity (FIG. 6C). To further test the idea that the amino **terminal** region of **p53** was required for MDM2 binding, we generated an additional yeast strain expressing the lexA-DNA binding domain fused to **p53** codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-**terminus of p53** were required for the interaction.

Detailed Description Text - DETX (47):

Sequence analysis showed that all **p53 and MDM2 fragments** noted in FIG. 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in FIG. 6 expressed the relevant proteins at similar levels, as shown by Western blotting (FIG. 7).

Claims Text - CLTX (1):

1. A polypeptide consisting of a contiguous **portion of p53, said portion comprising amino acids 13-41 of p53** as shown in SEQ ID NO:1, said polypeptide capable of binding to human MDM2.

Other Reference Publication - OREF (5):

Hinds, et al., "Mutant **p53 DNA Clones From Human** Colon Carcinomas Cooperate With Ras in Transforming Primary Rat Cells: A Comparison of the Hot Spot Mutant Phenotypes", Cell Growth & Differentiation, 1:561-580(1990).

Other Reference Publication - OREF (8):
    Oliner, et al., "Amplification of a Gene Encoding a **p53-Associated Pr tein in Human** Sarcomas", Nature, 358:80-83(1992).

US-PAT-NO: 5702903

DOCUMENT-IDENTIFIER: US 5702903 A

TITLE: Method and cells for drug identification

DATE-ISSUED: December 30, 1997

INVENTOR-INFORMATION:
NAME   CITY   STATE   ZIP CODE COUNTRY
Kinzler; Kenneth W.   Baltimore   MD   N/A   N/A
Vogelstein; Bert   Baltimore   MD   N/A   N/A

APPL-NO: 08/ 557393

DATE FILED: November 13, 1995

PARENT-CASE:

   This is a division of application Ser. No. 08/245,500 (issued as U.S. Pat.
No. 5,550,023) filed on May 18, 1994, which is a divisional of Ser. No.
08/044,619 (issued as U.S. Pat. No. 5,420,263) filed on Apr. 7, 1993, which
is a continuation-in-part of Ser. No. 07/903,103 (issued as U.S. Pat. No.
5,411,860) filed Jun. 23, 1992, which is a continuation-in-part of Ser. No.
07/867,840 (abandoned) filed Apr. 7, 1992.

US-CL-CURRENT: 435/6, 435/254.2 , 435/29 , 435/325 , 435/7.1

ABSTRACT:

   A human gene has been discovered which is genetically altered in human tumor
cells. The genetic alteration is gene amplification and leads to a
corresponding increase in gene products. Detecting that the gene, designated
hMDM2, has become amplified or detecting increased expression of gene products
is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and
allows the cell to escape from p53-regulated growth. Methods of identifying
compounds which interfere with the binding of **human MDM2 protein to human p53**
protein involved host cells which contain p-53-responsive reporter constructs
and MDM2 polypeptides.

4 Claims, 24 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 18

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Abstract Text - ABTX (1):

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and allows the cell to escape from p53-regulated growth. Methods of identifying compounds which interfere with the binding of **human MDM2 protein to human p53** protein involved host cells which contain p-53-responsive reporter constructs and MDM2 polypeptides.

Brief Summary Text - BSTX (6):

While there exists an enormous body of evidence linking **p53 gene mutations to human** tumorigenesis (Hollstein et al., 1991, Science 253:49-53) little is known about cellular regulators and mediators of p53 function.

Brief Summary Text - BSTX (7):

Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated ras oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253:49-53. The transformed fibroblasts were found to produce elevated levels of **human p53** protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Brief Summary Text - BSTX (8):

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type **human p53** gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

Brief Summary Text - BSTX (9):

Hinds et al., supra also expressed **human p53** protein in transformed rat cells. When the expressed **human p53** was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified M.sub.r 90,000 protein was coimmunoprecipitated. This suggested that the rat M.sub.r 90,000 protein is in a complex with the **human p53** protein in the transformed rat cell line.

Brief Summary Text - BSTX (19):

Yet another object of the invention is to provide methods for identifying

compounds which interfere with the binding of **human MDM2 t human p53**.

Brief Summary Text - BSTX (22):
   Still another object of the invention is to provide polypeptides which
interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (24):
   It has now been discovered that hMDM2, a heretofore unknown human gene,
plays a role in human cancer. The hMDM2 gene has been cloned and the
recombinant derived hMDM2 protein shown to bind to **human p53** in vitro. hMDM2
has been found to be amplified in some neoplastic cells and the expression of
hMDM2-encoded products has been found to be correspondingly elevated in tumors
with amplification of this gene. The elevated levels of MDM2 appear to
sequester p53 and allow the cell to escape from p53-regulated growth.

Drawing Description Text - DRTX (14):
   FIG. 6C. Random **fragments of p53** were fused to the sequence encoding the
B42 acidic activation domain and a hemagglutinin epitope tag; the resultant
clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain
fused to full length MDM2) and pJK103. Yeast clones were identified as above,
and all were found to be MDM2-dependent. The bottom three clones were
generated by genetic engineering.

Detailed Description Text - DETX (13):
   It has been found that amino acid residues 13-41 of **p53** (See SEQ ID NO: 1)
are necessary for the interaction of MDM-2 and **p53**. However, additional
residues on either the amino or carboxy **terminal** side of the **peptide** appear
also to be required. Nine to 13 additional **p53** residues are sufficient to
achieve MDM2 binding, although less may be necessary. Since cells which
overexpress MDM2 escape from **p53**-regulated growth control in sarcomas, the use
of **p53-derived peptides to bind to excess MDM2 leads to reestablishment of
p53**-regulated growth control.

Detailed Description Text - DETX (14):
   Suitable **p53-derived peptides** for administration are those which are
circular, linear, or derivitized to achieve better penetration of membranes,
for example. Other organic compounds which are modelled to achieve the same
three dimensional structure as the peptide of the invention can also be used.

Detailed Description Text - DETX (15):
   DNA encoding the MDM2-binding, **p53-derived peptide,** or multiple copies
thereof, may also be administered to tumor cells as a mode of administering the
peptide. The DNA will typically be in an expression construct, such as a
retrovirus, DNA virus, or plasmid vector, which has the DNA elements necessary
for expression properly positioned to achieve expression of the MDM2-binding
peptide. The DNA can be administered, inter alia encapsulated in liposomes, or

in any other form known to the an to achieve efficient uptake by cells. As in the direct administration of **peptide, the goal is to alleviate the sequestration of p53** by MDM2.

Detailed Description Text - DETX (18):

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to **human p53**. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

Detailed Description Text - DETX (27):

To determine whether the hMDM2 protein could bind to **human p53** protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in FIG. 1 from nucleotide 312 to 2176. A 42 bp black beetle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Detailed Description Text - DETX (33):

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (FIG. 2, lanes 2 and 3). However, when in vitro translated **human p53** was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association in vitro (FIG. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (FIG. 2, lanes 8 and 9). When an in vitro translated mutant form of p53 (175.sup.his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

Detailed Description Text - DETX (60):

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA **fragments encoding MDM2 or p53** were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

Detailed Description Text - DETX (62):

Full length MDM2 cDNA in pBluescript SK+ (Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K. W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D. M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK 103 (lexA-responsive .beta.-galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2 % dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent clones are diagrammed in FIG. 6A. The MDM2 sequences of the remaining 15 **p53-dependent clones coded for peptides** ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine: Three of the MDM2 sequences obtained are shown at the top of FIG. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Detailed Description Text - DETX (63):

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-**terminal** to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three **p53** sequences shown in FIG. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated **fragments**.

Detailed Description Text - DETX (64):

The resultant yeast colonies were examined for .beta.-galactosidase activity in situ. Of approximately 5000 clones containing MDM2 **fragments** fused to the lexA DNA binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of .beta.-galactosidase (about 5-fold less than the other fifteen clones) and .beta.-galactosidase expression was independent of **p53** expression (FIG. 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301 (SEQ ID NO:3)). This domain consisted of 37.5 % aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable .beta.-galactosidase activity in the same assay

(Table I, strain 3). The other class (15 clones) each contained the amino **terminal** region of MDM2 (FIG. 6B). The .beta.-galactosidase activity of these clones was dependent on **p53** co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length **p53** contained MDM2 codons 1 to 118 (FIG. 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated **fragments** of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

Detailed Description Text - DETX (65):

In a converse set of experiments, yeast clones containing **fragments of p53** fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (FIG. 6C). The minimal overlap between these three **fragments** contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the **fragments** required 9-12 amino acids on either side of codons 13-41 for activity (FIG. 6C). To further test the idea that the amino **terminal** region of **p53** was required for MDM2 binding, we generated an additional yeast strain expressing the lexA-DNA binding domain fused to **p53** codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-**terminus of p53** were required for the interaction.

Detailed Description Text - DETX (66):

Sequence analysis showed that all **p53 and MDM2 fragments** noted in FIG. 6 were ligated in frame and in the correct orientation relative to the 1342 and lexA domains, respectively. Additionally, all clones compared in FIG. 6 expressed the relevant proteins at similar levels, as shown by Western blotting (FIG. 7).

Claims Text - CLTX (1):

1. A method for identifying compounds which interfere with the binding of **human MDM2 to human p53,** comprising the steps of:

Claims Text - CLTX (3):

(a) expresses an MDM2 polypeptide and a **p53 polypeptide, wherein each of said polypeptides contains a sufficient portion of p53** or MDM2 proteins to bind to the other polypeptide;

Claims Text - CLTX (11):

2. A method of identifying compounds which interfere with the binding of **human MDM2 t human p53,** comprising:

Claims Text - CLTX (12):
   providing a cell which comprises three recombinant DNA constructs, said
first construct encoding a first polypeptide fused to a sequence-specific
DNA-binding domain, said second contact encoding a second polypeptide fused to
a transcriptional activation domain, said third construct comprising a reporter
gene downstream from a DNA element which is recognized by said
sequence-specific DNA-binding domain, wherein said first polypeptide is an MDM2
polypeptide and said second protein is a p53 polypeptide, or said first
polypeptide is a p53 polypeptide and said second polypeptide is an MDM2
polypeptide; wherein said polypeptides contain a sufficient **portion of p53** and
MDM2 proteins to bind to the other polypeptide;

Claims Text - CLTX (16):
   (a) expresses an MDM2 polypeptide and a **p53 polypeptide, wherein each of
said polypeptides contains a sufficient portion of p53** or MDM2 proteins to bind
to the other polypeptide;

Claims Text - CLTX (22):
   4. A cell which comprises three recombinant DNA constructs, said first
construct encoding a first polypeptide fused to a sequence-specific DNA-binding
domain, said second construct encoding a second polypeptide fused to a
transcriptional activation domain, said third construct comprising a reporter
gene downstream from a DNA element which is recognized by said
sequence-specific DNA-binding domain, wherein said first polypeptide is an MDM2
polypeptide and said second protein is a p53 polypeptide, or said first
polypeptide is a p53 polypeptide and said second polypeptide is an MDM2
polypeptide; wherein said polypeptides contain a sufficient **portion of p53** and
MDM2 proteins to bind to the other polypeptide.

Other Reference Publication - OREF (2):
   Hinds, et al., "Mutant **p53 DNA Clones From Human** Colon Carcinomas Cooperate
With Ras in Transforming Primary Rat Cells: A Comparison of the Hot Spot Mutant
Phenotypes", Cell Growth & Differentiation, 1:561-580 (1990).

Other Reference Publication - OREF (5):
   Oliner, et al, "Amplification of a Gene Encoding a **p53-Associated Protein in
Human** Sarcomas", Nature, 358:80-83 (1992).

Other Reference Publication - OREF (7):
   Leach, et al., "**p53 Mutation and MDMS Amplification in Human** Soft Tissue
Sarcomas", Cancer Research 53:2231-2234 (1993).

US-PAT-NO: 5688918

DOCUMENT-IDENTIFIER: US 5688918 A

TITLE: p53as protein and antibody therefor

DATE-ISSUED: November 18, 1997

INVENTOR-INFORMATION:
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Kulesz-Martin; Molly F.   Buffalo   NY   N/A   N/A

APPL-NO: 08/ 259612

DATE FILED: June 14, 1994

PARENT-CASE:

BACKGROUND OF THE INVENTION

This is a continuation-in-part of U.S. patent application Ser. No.
08/195,952 filed Feb. 14, 1994 which is a continuation in part of U.S.
application Ser. No. 08/100,496, filed Aug. 2, 1993.

US-CL-CURRENT: 530/387.7, 530/388.8 , 530/388.85 , 530/389.7

ABSTRACT:

The invention comprises plasmids and viral vectors containing an animal
p53as cDNA sequence. A portion of the p53as sequence may be identified to a
position of wild type p53 gene from the same animal. In preferred embodiments,
the p53as is mouse or human p53as. A preferred viral vector is baculovirus
vector. The invention further includes antibodies both polyclonal and
monoclonal, to p53as and to at least a **portion of human p53** intron 10 sequence
encoding SLRPFKALVREKGHRPSSHSC which is related to p53as sequences and plasmids
and viral vectors containing such sequences. All of the above find utility in
studying p53 and p53as and their relative expressions which is believed
important for detection and control of malignant cells and their susceptibility
to treatment agents. The antibodies can detect the presence of p53as and
related sequences and when injected into cells could cause cell cycle arrest
and the plasmids and viral vectors, with appropriate promotors, can cause
expression of the p53as and p53 intron 10 sequences which can affect cell
growth and perhaps arrest certain malignancies.

6 Claims, 21 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 9

Abstract Text - ABTX (1):

The invention comprises plasmids and viral vectors containing an animal
p53as cDNA sequence. A portion of the p53as sequence may be identified to a
position of wild type p53 gene from the same animal. In preferred embodiments,
the p53as is mouse or human p53as. A preferred viral vector is baculovirus
vector. The invention further includes antibodies both polyclonal and
monoclonal, to p53as and to at least a **portion of human p53** intron 10 sequence
encoding SLRPFKALVREKGHRPSSHSC which is related to p53as sequences and plasmids
and viral vectors containing such sequences. All of the above find utility in
studying p53 and p53as and their relative expressions which is believed
important for detection and control of malignant cells and their susceptibility
to treatment agents. The antibodies can detect the presence of p53as and
related sequences and when injected into cells could cause cell cycle arrest
and the plasmids and viral vectors, with appropriate promotors, can cause
expression of the p53as and p53 intron 10 sequences which can affect cell
growth and perhaps arrest certain malignancies.

Brief Summary Text - BSTX (4):

This application provides further further support for the utility of wild
type p53as expression from plasmids and vectors such as those described in
application Ser. No. 08/195,952. Further evidence is presented that p53as
protein has "tumor suppressor" activity in mouse and human cells, activates
transcription through **p53 target sequences of mouse and human** cells, and forms
tetramers, a DNA binding form observed for the tumor suppressor gene p53as.
This Application further claims the use of antibodies to human p53as for
diagnosis of human cancers, the use of antibodies to human p53as for
determination of prognosis of human cancers and use of antibodies to human
p53as for determining a treatment plan for individual patient cancers.

Brief Summary Text - BSTX (5):

As described in the parent applications, the invention also comprises
plasmids and viral vectors containing an animal p53as cDNA sequence. A portion
of the p53as sequence may be identified to a position of wild type p53 gene
from the same animal. In preferred embodiments, the p53as is mouse or human
p53as. A preferred viral vector is baculovirus vector. The invention further
includes antibodies both polyclonal and monoclonal, to p53as and to at least a
portion of human p53 intron 10 sequence encoding SLRPFKALVREKGHRPSSHSC (SEQ ID
NO.1) which is related to p53as sequences and plasmids and viral vectors
containing such sequences.

Drawing Description Text - DRTX (17):

FIGS. 10A, 10B and 10C show colony formation of Saos-2 cells transfected
with CMV plasmids. FIG. 10A shows a control without a cDNA insect. FIG. 10B
shows a p53as cDNA insect and FIG. 10C shows a p53 cDNA insect. Plasmid
containing p53as containing a CMV promoter (which drives expression of p53as in
the target mammalian cell) is introduced into **human osteosarcoma cells which**

lack p53 which stops tumor cell growth and reduces the number and area of colonies present in the culture dishes. This assay is commonly used to show tumor suppressor activity of various genes and p53 used as a control shows similar activity. thus wild type p53as acts as a tumor suppressor. In contrast, a plasmid containing an alternatively spliced mutant p53 sequence had transforming activity (Eliyahu et at., Oncogene 3,313-321, 1988.). Tumor suppressor activity was found in human cells as well as mouse cells. Human osteosarcoma cell line Saos-2 was trans fected using lipofectin (BRL0 with 5 ng of the pCMV plasmids containing the p53 or p53as cDNA indicated and a ncomycin resistance gene. Vector without the cDNA insert was used as a control. Forty-eight hours after transfection, cells were trypsinized, passaged at a 1:4 ratio and cultured in media containing 500 .mu.g/ml G418 for 3 weeks. Colonies were fixed in methanol and stained with Giemsa and measured using an image analysis system (Spectra Services).

Detailed Description Text - DETX (4):

   Plasmids containing the cDNA sequence unique to p53as are included in this invention. One such plasmid is pBSp53as which contains full length alternatively spliced **p53** cDNA. pBSp53as was constructed from **p53** cDNA beginning at nt-111 of the (where 1 is the first ATG encoding methionine) and ending at nt 1539, cloned into the EcoRI and BamHI sites of pBluescript SK under the control of a T3 phage promoter. The N-**terminal fragment of wt p53** was amplified by reverse transcriptase/polymerase chain reaction (RT-PCR) from a mouse epidermal cell RNA template and the C-**terminal fragment** of p53as was amplified by PCR from plasmid p6.4 (which contains an alternatively spliced **p53** cDNA; Han and Kulesz-Martin, Nucl. Acids Res. 20: 1979-81, 1992) using primers which contained a StuI restriction site at the 5'end of the sense primer

Detailed Description Text - DETX (6):

   and a BamHI site at the 5' end of the antisense primer. pBSp53 was made from pBSp53as by replacement of the StuI/BamHI C-**terminal fragment** of p53as cDNA with the StuI/BamHI segment of wild type **p53** cDNA from plasmid pLSVNc51 (ref. Oren). In particular, cDNA for the N-**terminus of p53** (nt -111 to 1090) was made using template RNA from 291 nontransformed epidermal cells by means of a reverse transcriptase reaction, amplified by PCR and cloned into the EcoRI and BamHI sites of pBluescript SK under the control of a T3 phage promoter to create plasmid pBSRS13. The primers used for PCR were: sense, AGTCGAATTCATTGGGACCATCCTGGCT (SEQ ID NO.3), antisense, AGTCGGATCCTGGAGTGAGTGAGCCCTGCTGTCT (SEQ ID NO.4). These primers contained an EcoRI restriction site at the 5' end of the sense primer and a BamHI site at the 5' end of the antisense primer (denoted by underlining). The C-**terminal p53** cDNA (nt1028 to 1539) was amplified by PCR from plasmid p6.4 (which contains an alternatively spliced **p53** cDNA) using primers which contained a StuI restriction site at the 5' end of the sense primer

Detailed Description Text - DETX (8):

   and a BamHI site at the 5' end of the antisense primer (as in Han and

Kulesz-Martin, Nucl. Acids Res. 20: 1979-81, 1992). The StuI to BamHI
segment of this PCR reaction product was then ligated to the StuI and BamHI
sites of plasmid pBSRS13 to create plasmid pBSp53as, containing a full length
alternatively spliced p53 cDNA. To construct pBSp53, the StuI and BamHI
fragment fr m wt p53 cDNA was substituted for the StuI and BamHI fragment of
the p53as cDNA in pBSp53as.

Detailed Description Text - DETX (20):
    In contrast to cotranslation of p53 and p53as proteins, mixture of lysates
containing each protein translated individually did not show inactivation of
p53as by p53 protein for DNA binding, and PAb421 supershifted only one band,
suggesting that p53 and p53as must be translated together for association to
occur (FIG. 6). This is consistent with the report that oligomerization
between **human p53** and mouse p53 occurs when they are cotranslated, but not when
mixed (Milner and Medcalf, 1991).

Detailed Description Text - DETX (29):
    Methods were per manufacturers instructions (Invitrogen) and materials and
included linear AcMNPV DNA and transfer vector (e.g. pVL 1392, pVL 1393) and
insect cell line S. frugiperda Sf9, propagated at 27 C. in Grace's supplemented
insect medium containing 10% fetal bovine serum (GIBCO) and 10 ug/ml gentamycin
sulfate. pVL1393BGB53 baculovirus vector containing wt **p53 cDNA was
constructed by inserting the BglII/BamHI fragment of pLSVNc51 (including the
entire wt p53** cDNA) into the pVL 1393 vector. pVL1393Asp baculovirus vector
(containing p53as cDNA) was constructed by replacement of the StuI/BamHI
C**-terminal fragment** of p53as cDNA (Han and Kulesz-Martin, Nucl. Acids Res.
1992) with the BamHI/StuI **fragment** of the pVL1393BGB53 vector (see above). To
purify recombinant viruses, Sf9 cells were cotransfected with linear AcMNPV DNA
and the transfer vector containing p53as and grown for 3 days. Recombinant
viruses were identified by plaque assays or serial dilutions. Alternatively,
p53 and p53as baculovirus contructions will be cotransfected with linearized
(PharMingen) virus DNA which allows propagation of only recombinant virus.
Virus stocks which resulted in p53as expression in insect cells (assayed by
immunoblotting using anti-p53as antibody) were expanded and used to infect
insect cells for the flow cytometry studies.

Detailed Description Text - DETX (45):
    Because **human and mouse p53** proteins form complexes in cells, the construct
containing mouse p53as cDNA is claimed for the purposes of gene expression in
mammalian cells and nonmammalian cells for research purposes, including human
cells, and for gene therapy in humans. In addition, a purified plasmid
construct containing the human p53as homologue of mouse p53as, defined by
insertion of human intron 10 sequences into a sequence containing wt p53 DNA
(as defined in original patent application Ser. No. 08/100,486) is claimed for
research purposes in mammalian and nonmammalian cells, and for gene therapy in
humans.

Detailed Description Text - DETX (46):

Table 1. shows reactivities of antibodies against p53 proteins. Mouse **p53 has 390 amin acids; human p53** 393 amino acids. All antibodies are mouse monoclonals commercially available from Oncogene Science, Cambridge Mass., except ApAs rabbit polyclonal specific for p53as protein which was made in Dr. Kulesz-Martin's laboratory, RPCI. Sources: Oncogene Science Catalogue, p. 8, 1992; Vajtesek et al., J. of Immunolog. Methods 151:237-244, 1992, .sup.a Wade-Evans, A. and Jenkins, J. R. EMBO J., 4:699-706, 1985, .sup.b Gannon, EMBO, 9:1595-1602, 1990, .sup.c Stephen, C. W. and Lane, D. P., J. Mol. Biol., 5:577-583, 1992 and .sup.d Kulesz-Martin et al., Mol. Cell. Biol., in press, March 1994.

Detailed Description Text - DETX (50):
    Tables 3 and 4 demonstrate that p53as has transcriptional activity. Plasmids containing p53as, or **p53 as a control, were introduced into mouse or human** cells along with a plasmid containing a p53 binding site upstream of a reporter gene. The reporter was activated by p53as, with p53 as a positive control, but not by the vector without p53as. By comparison, the background activity of the reporter plasmid alone was low. The reporter sequences 50-2 and PC13 are a promoter-enhancer sequence (Zambetti et al., Genes Dev. 6, 1143-1152, 1992) and a consensus p53 binding sequence (Kern et al., Science 256, 827-830, 1992). p53 as was active on mouse (50-2) or **human (PG13) p53** binding sequences, and in both mouse and human cells.

Detailed Description Text - DETX (54):
    For example, tumor cells which express different ratios of p53as and p53, as detected at the RNA level, or at the protein level by anti-p53as antibodies may have different characteristics or different sensitivity to anti-cancer treatments. Different characteristics identifiable by reactivity with p53as antibodies could aid in the diagnosis of cancer, prognosis for individual patients with tumors and decisions about treatment based on the competency of the p53as functions in the tumor. Antibodies to **p53 are being used clinically in dianosis of human cancers because expression of p53** at detectable levels has been found to correlate with cancer prognosis for a variety of human tumor types. It is known that tumors with defective **p53 genes (more than 50% of all human** cancers) fail to control cell cycle progesssion normally. Since p53 and p53as have different cell cycle associations, tumor typing using specific p53as antibodies and specific anti-p53 antibidies may increase the value of typing individual tumors according to their expression of tumor suppressor gene products such as p53. Such tumor typing may provide useful information in the diagnosis, prognosis, and treatment strategy of individual patient cancers.

Detailed Description Paragraph Table - DETL (1):
    TABLE 1

Reactivities of Antibodies Against p53 Proteins wt wt cell frozen paraffin
Ab Species conform denatured mutated p53 p53as epitope IP WIB staining sections sections

PAb421.sup.a mu/hu + + + + - 370-378 + +/- + + - PAb246.sup.a mu + - - +

+ 88-109 + - + + nt PAb240.sup.bc mu/hu - + + + (-) 210-214 + + + + -
ApAs.sup.d mu + + nt - + (364-381) + + + nt nt

Mouse **p53 has 390 amino acids; human p53** 393 amino acids. All antibodies are mouse monoclonals commercially available from Oncogene Science, Cambridge MA, except ApAs rabbit polyclonal specific for p53as protein which was made in Dr. KuleszMartin's laboratory, RPCO. Sources: Oncogene Science Catalogue, p. 8, 1992; Vajtesek et al., J. of Immunolog. Methods 151:237-244, 1992, .sup.a WadeEvans, A. and Jenkins, J. R. EMBO J., 4:699-706, 1985, .sup.b Gannon, EMBO, 9:1595-1602, 1990, .sup.c Stephen, C. W. and Lane, D. P., J. Mol. Biol., 5:577-583, 1992 and .sup.d KuleszMartin et al., Mol. Cell. Biol., in press, March 1994.

Claims Text - CLTX (1):
   1. An antibody which specifically binds to mammalian p53as protein and does not bind to normal **p53 from the same species wherein said antibody binds to an epitope present in a peptide** unique to p53as, said **peptide** occurring within the final 50 carboxyl **terminal** amino acids of p53as.

Other Reference Publication - OREF (19):
   Janice M. Nigro et al., "**Human p53** and CDC2Hs Genese Combine to Inhibit the Proliferation of Saccharomyces cerevisiae", Molecular and Cellular Biology, (1992), pp. 1357-1365.

Other Reference Publication - OREF (33):
   James R. Bischoff et al., "**Human p53** Inhibits Growth in Schizosaccharomyces pombe", Molecular and Cellular Biology, (1992) vol. 12, No. 4, pp. 1405-1411.

Other Reference Publication - OREF (36):
   Jill Bargonetti et al, "A Proteolytic **Fragment From the Central Region of p53** Has Marked Sequence-Specific DNA-Binding Activity When Generated From Wild-Type But Not From Oncogenic Mutant p53 Protein", Genes & Development, 7, (1993) pp. 2565-2574.

Other Reference Publication - OREF (39):
   Walter D. Funk et al., "A Transcriptionally Active DNA-Binding Site for **Human p53** Protein Complexes", Mol. and Cell. Bio., (1992) pp. 2866-2871.

Other Reference Publication - OREF (43):
   J.D. Oliner et al., "Amplification of a Gene Encoding a **p53-Associated Protein in Human** Sarcomas", Nature, vol. 358, (1992), pp. 80-83.

Other Reference Publication - OREF (51):
   B. Vojtesek et al., "An Immunochemical Analysis of the **Human Nuclear Phosphopr tein p53**", Journal of Immunological Methods, 151 (1992) pp. 237-244.

US-PAT-NO: 5618921

DOCUMENT-IDENTIFIER: US 5618921 A
See image for Certificate of Correction

TITLE: Antibodies for detection of human MDM2 protein

DATE-ISSUED: April 8, 1997

INVENTOR-INFORMATION:

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Hill; David E.	Arlington	MA	N/A	N/A
Kinzler; Kenneth W.	Baltimore	MD	N/A	N/A
Vogelstein; Bert	Baltimore	MD	N/A	N/A

APPL-NO: 08/ 390479

DATE FILED: February 17, 1995

PARENT-CASE:

   This application is a division of application Ser. No. 08/044,619 filed
Apr. 7, 1993, now U.S. Pat. 5,420,263, which is a continuation-in-part of
Ser. No. 07/903,103, filed Jun. 23, 1992, now U.S. Pat. No. 5,411,619,
which is a continuation in part of Ser. No. 07/867,840 filed Apr. 7, 1992,
abandoned.

US-CL-CURRENT: 530/387.7, 435/330 , 435/344.1 , 435/7.1 , 530/350
          , 530/388.85 , 530/389.7

ABSTRACT:

   A human gene has been discovered which is genetically altered in human tumor
cells. The genetic alteration is gene amplification and leads to a
corresponding increase in gene products. Detecting that the gene, designated
hMDM2, has become amplified or detecting increased expression of gene products
is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and
allows the cell to escape from p53-regulated growth. Antibodies to the human
MDM2 protein and kits containing MDM2 specific antibodies are disclosed.

15 Claims, 24 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 17

--------- KWIC ---------

Abstract Text - ABTX (1):

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and allows the cell to escape from p53-regulated growth. Antibodies to the human MDM2 protein and kits containing MDM2 specific antibodies are disclosed.

Brief Summary Text - BSTX (6):

While there exists an enormous body of evidence linking **p53 gene mutations to human** tumorigenesis (Hollstein et al., 1991, Science 253:49-53) little is known about cellular regulators and mediators of p53 function.

Brief Summary Text - BSTX (7):

Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated ras oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253:49-53. The transformed fibroblasts were found to produce elevated levels of **human p53** protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Brief Summary Text - BSTX (8):

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type **human p53** gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 481-96.

Brief Summary Text - BSTX (9):

Hinds et al., supra also expressed **human p53** protein in transformed rat cells. When the expressed **human p53** was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified M.sub.r 90,000 protein was coimmunoprecipitated. This suggested that the rat M.sub.r 90,000 protein is in a complex with the **human p53** protein in the transformed rat cell line.

Brief Summary Text - BSTX (19):

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (22):
   Still another object of the invention is to provide polypeptides which
interfere with the binding of **human MDM2 t   human p53**.

Brief Summary Text - BSTX (24):
   It has now been discovered that hMDM2, a heretofore unknown human gene,
plays a role in human cancer. The hMDM2 gene has been cloned and the
recombinant derived hMDM2 protein shown to bind to **human p53** in vitro. hMDM2
has been found to be amplified in some neoplastic cells and the expression of
hMDM2-encoded products has been found to be correspondingly elevated in tumors
with amplification of this gene. The elevated levels of MDM2 appear to
sequester p53 and allow the cell to escape from p53-regulated growth.

Drawing Description Text - DRTX (13):
   FIG. 6C. Random **fragments of p53** were fused to the sequence encoding the
1342 acidic activation domain and a hemagglutinin epitope tag; the resultant
clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain
fused to full length MDM2) and pJK103. Yeast clones were identified as above,
and all were found to be MDM2-dependent. The bottom three clones were
generated by genetic engineering.

Detailed Description Text - DETX (13):
   It has been found that amino acid residues 13-41 of **p53** (See SEQ ID NO: 1)
are necessary for the interaction of MDM-2 and **p53**. However, additional
residues on either the amino or carboxy **terminal** side of the **peptide** appear
also to be required. Nine to 13 additional **p53** residues are sufficient to
achieve MDM2 binding, although less may be necessary. Since cells which
overexpress MDM2 escape from **p53**-regulated growth control in sarcomas, the use
of **p53-derived peptides to bind to excess MDM2 leads to reestablishment of
p53**-regulated growth control.

Detailed Description Text - DETX (14):
   Suitable **p53-derived peptides** for administration are those which are
circular, linear, or derivitized to achieve better penetration of membranes,
for example. Other organic compounds which are modelled to achieve the same
three dimensional structure as the peptide of the invention can also be used.

Detailed Description Text - DETX (15):
   DNA encoding the MDM2-binding, **p53-derived peptide,** or multiple copies
thereof, may also be administered to tumor cells as a mode of administering the
peptide. The DNA will typically be in an expression construct, such as a
retrovirus, DNA virus, or plasmid vector, which has the DNA elements necessary
for expression properly positioned to achieve expression of the MDM2-binding
peptide. The DNA can be administered, inter alia encapsulated in liposomes, or
in any other form known to the art to achieve efficient uptake by cells. As in

the direct administration of **peptide, the goal is to alleviate the sequestration of p53** by MDM2.

Detailed Description Text - DETX (18):

   The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to **human p53**. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery, allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

Detailed Description Text - DETX (27):

   To determine whether the hMDM2 protein could bind to **human p53** protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in FIG. 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (E1-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Detailed Description Text - DETX (33):

   The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (FIG. 2, lanes 2 and 3). However, when in vitro translated **human p53** was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association in vitro (FIG. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (FIG. 2, lanes 8 and 9). When an in vitro translated mutant form of p53 (175.sup.his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

Detailed Description Text - DETX (59):

   This assay was then applied to mapping the interaction domains of each protein. Full length cDNA **fragments encoding MDM2 or p53** were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

Detailed Description Text - DETX (60):

   METHODS. Full length MDM2 cDNA in pBluescript SK +(Stratagene) was digested

with XhoI and BamHI to excise the entire insert. After agarose gel
purification, the insert was sheared into random fragments by sonication,
polished with the Klenow fragment of DNA polymerase I, ligated to catch
linkers, and amplified by the polymerase chain reaction as described (Kinzler,
K. W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were
fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000
pb, cloned into lexA(1-202)+PL (Ruden, D. M., et al., Nature 350:250-252
(1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least
10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was
transfected into a strain of pEGY48 containing pRS3 14N (p53 expression vector)
and pIK103 (lexA-responsive .beta.-galactosidase reporter). Approximately
5,000 yeast clones were plated on selective medium containing 2 % dextrose, and
were replica-plated onto glalctose- and X-gal-containing selective medium.
Blue colonies (17) appeared only on the plates containing the larger fragments
of MDM2. The 17 isolated colonies were tested for blue color in this assay
both in the presence and in the absence of galactose (p53 induction); all
tested positive in the presence of galactose but only 2 of the 17 tested
positive in its absence. MDM2-containing plasmid DNA extracted from the 17
yeast clones was selectively transferred to bacterial strain KC8 and sequenced
from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent
clones are diagrammed in FIG. 6A. The MDM2 sequences of the remaining 15
p53-dependent clones coded for peptides ranging from 135 to 265 a.a. in length
and began exclusively at the initiator methionine. Three of the MDM2 sequences
obtained are shown at the top of FIG. 6B. The lower 6 sequences were
genetically engineered (using the polymerase chain reaction and appropriate
primers) into lexA(1-202)+PL and subsequently tested to further narrow the
binding region.

Detailed Description Text - DETX (61):
   Fragments of p53 were also cloned into pJG4-5, producing a fusion protein
C-**terminal** to the 1342 acidic activation domain and incorporating an epitope of
hemagglutinin. The clones were transfected into a strain of pEGY48 already
containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The
top three **p53** sequences shown in FIG. 6C. were derived from yeast obtained by
colony screening, whereas the lower three were genetically engineered to
contain the indicated **fragments**.

Detailed Description Text - DETX (62):
   The resultant yeast colonies were examined for .beta.-galactosidase activity
in situ. Of approximately 5000 clones containing MDM2 **fragments** fused to the
lexA DNA binding domain, 17 were found to score positively in this assay. The
clones could be placed into two classes. The first class (two clones)
expressed low levels of .beta.-galactosidase (about 5-fold less than the other
fifteen clones) and .beta.-galactosidase expression was independent of **p53**
expression (FIG. 6A). These two clones encoded MDM2 amino acids 190-340 and
269-379, respectively. The region shared between these two clones overlapped
the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of
37.5 % aspattic and glutamic acid residues but no basic amino acids. This
acidic domain appears to activate transcription only when isolated from the
rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no
measurable .beta.-galactosidase activity in the same assay (Table I, strain 3).

The other class (15 clones) each contained the amino **terminal** region of MDM2 (FIG. 6B). The .beta.-galactosidase activity of these clones was dependent on **p53** co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length **p53** contained MDM2 codons 1 to 118 (FIG. 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated **fragments** of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

Detailed Description Text - DETX (63):

In a converse set of experiments, yeast clones containing **fragments of p53** fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (FIG. 6C). The minimal overlap between these three **fragments** contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the **fragments** required 9-12 amino acids on either side of codons 13-41 for activity (FIG. 6C). To further test the idea that the amino **terminal** region of **p53** was required for MDM2 binding, we generated an additional yeast strain expressing the lexA-DNA binding domain fused to **p53** codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-**terminus of p53** were required for the interaction.

Detailed Description Text - DETX (64):

Sequence analysis showed that all **p53 and MDM2 fragments** noted in FIG. 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in FIG. 6 expressed the relevant proteins at similar levels, as shown by Western blotting (FIG. 7).

Other Reference Publication - OREF (2):

Hinds, et al., "Mutant **p53 DNA Clones From Human** Colon Carcinomas Cooperate With Ras in Transforming Primary Rat Cells: A Comparison of the Hot Spot Mutant Phenotypes", Cell Growth & Differentiation, 1:561-580 (1990).

Other Reference Publication - OREF (5):

Oliner, et al., "Amplification of a Gene Encoding a **p53-Associated Protein in Human** Sarcomas", Nature, 358:80-83 (1992).

Other Reference Publication - OREF (7):

Leach, et al., "**p53 Mutation and MDMS Amplification in Human** Soft Tissue Sarcomas", Cancer Research 53:2231-2234 (1993).

US-PAT-NO: 5606044

DOCUMENT-IDENTIFIER: US 5606044 A

TITLE: Kits for detecting amplification of human MDM2

DATE-ISSUED: February 25, 1997

INVENTOR-INFORMATION:

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Kinzler; Kenneth W.	Baltimore	MD	N/A	N/A
Vogelstein; Bert	Baltimore	MD	N/A	N/A

APPL-NO: 08/ 390546

DATE FILED: February 17, 1995

PARENT-CASE:

This application is a continuation of application Ser. No. 08/044,619 (allowed) filed 04/07/93 which is issued as U.S. Pat. No. 5,420,263, which is a continuation-in-part of Ser. No. 07/903,103 (allowed) filed Jun. 23, 1992, which issued as U.S. Pat. No. 5,411,860, which is a continuation-in-part of Ser. No. 07/867,840 (abandoned) filed Apr. 7, 1992 .

US-CL-CURRENT: 536/24.31, 206/569

ABSTRACT:

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and allows the cell to escape from p53-regulated growth.

2 Claims, 24 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 17

---------- KWIC ---------

Abstract Text - ABTX (1):
A human gene has been discovered which is genetically altered in human tumor

cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and allows the cell to escape from p53-regulated growth.

Brief Summary Text - BSTX (6):
   While there exists an enormous body of evidence linking **p53 gene mutations to human** tumorigenesis (Hollstein et al., 1991, Science 253:49-53) little is known about cellular regulators and mediators of p53 function.

Brief Summary Text - BSTX (7):
   Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated ras oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253:49-53. The transformed fibroblasts were found to produce elevated levels of **human p53** protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Brief Summary Text - BSTX (8):
   Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type **human p53** gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

Brief Summary Text - BSTX (9):
   Hinds et al., supra also expressed **human p53** protein in transformed rat cells. When the expressed **human p53** was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified M.sub.r 90,000 protein was coimmunoprecipitated. This suggested that the rat M.sub.r 90,000 protein is in a complex with the **human p53** protein in the transformed rat cell line.

Brief Summary Text - BSTX (19):
   Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (22):
   Still another object of the invention is to provide polypeptides which

interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (24):

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to **human p53** in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

Drawing Description Text - DRTX (7):

FIG. 6 shows the determination of MDM2 and p53 domains of interaction. FIG. 5A and FIG. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS3145SN (p53 expression vector) and pJK103 (lexA-responsive .beta.-galactosidase reporter). Yeast clones expressing .beta.-galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined. .beta.-galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. FIG. 6C. Random **fragments of p53** were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Detailed Description Text - DETX (13):

It has been found that amino acid residues 13-41 of **p53** (See SEQ ID NO: 1) are necessary for the interaction of MDM-2 and **p53**. However, additional residues on either the amino or carboxy **terminal** side of the **peptide** appear also to be required. Nine to 13 additional **p53** residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from **p53**-regulated growth control in sarcomas, the use of **p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53**-regulated growth control.

Detailed Description Text - DETX (14):

Suitable **p53-derived peptides** for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

Detailed Description Text - DETX (15):

DNA encoding the MDM2-binding, **p53-derived peptide,** or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a

retrovirus, DNA virus, or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, inter alia encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of **peptide, the goal is to alleviate the sequestration of p53** by MDM2.

Detailed Description Text - DETX (18):

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to **human p53**. Moreover, it has been found that hMDM2 is amplified in some satcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

Detailed Description Text - DETX (27):

To determine whether the hMDM2 protein could bind to **human p53** protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in FIG. 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 5:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Detailed Description Text - DETX (33):

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (FIG. 2, lanes 2 and 3). However, when in vitro translated **human p53** was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association in vitro (FIG. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kintier et al., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (FIG. 2, lanes 8 and 9). When an in vitro translated mutant form of p53 (175.sup.his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

Detailed Description Text - DETX (59):

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA **fragments encoding MDM2 or p53** were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

Detailed Description Text - DETX (60):

METHODS. Full length MDM2 cDNA in pBluescript SK+ (Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K. W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D. M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive .beta.-galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gat-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galaclose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent clones are diagrammed in FIG. 6A. The MDM2 sequences of the remaining 15 **p53-dependent clones coded for peptides** ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of FIG. 6B. The lower 6 sequences were genetically engineer (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Detailed Description Text - DETX (61):

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-**terminal** to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three **p53** sequences shown in FIG. 6C were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated **fragments**.

Detailed Description Text - DETX (62):

The resultant yeast colonies were examined for .beta.-galactosidase activity in situ. Of approximately 5000 clones containing MDM2 **fragments** fused to the lexA DNA binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of .beta.-galactosidase (about 5-fold less than the other fifteen clones) and .beta.-galactosidase expression was independent of **p53** expression (FIG. 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of

37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no-measurable galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino **terminal** region of MDM2 (FIG. 6B). The .beta.-galactosidase activity of these clones was dependent on **p53** co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length **p53** contained MDM2 codons 1 to 118 (FIG. 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated **fragments** of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

Detailed Description Text - DETX (63):

In a converse set of experiments, yeast clones containing **fragments of p53** fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (FIG. 2C). The minimal overlap between these three **fragments** contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the **fragments** required 9-12 amino acids on either side of codons 13-41 for activity (FIG. 6C). To further test the idea that the amino **terminal** region of **p53** was required for MDM2 binding, we generated an additional yeast strain expressing the lexA-DNA binding domain fused to **p53** codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-**terminus of p53** were required for the interaction.

Detailed Description Text - DETX (64):

Sequence analysis showed that all **p53 and MDM2 fragments** noted in FIG. 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in FIG. 6 expressed the relevant proteins at similar levels, as shown by Western blotting (FIG. 7).

Other Reference Publication - OREF (2):

Hinds, et al., "Mutant **p53 DNA Clones From Human** Colon Carcinomas Cooperate With Ras in Transforming Primary Rat Cells: A Comparison of the Hot Spot Mutant Phenotypes", Cell Growth & Differentiation, 1:561-580 (1990).

Other Reference Publication - OREF (5):

Oliner, et al., "Amplification of a Gene Encoding a **p53-Associated Protein in Human** Sarcomas", Nature, 358:80-83 (1992).

Other Reference Publication - OREF (7):
    Leach, et al., "**p53 Mutation and MDMS Amplification in Human** Soft Tissue
Sarcomas", Cancer Research 53:2231-2234 (1993).

US-PAT-NO: 5569824

DOCUMENT-IDENTIFIER: US 5569824 A

TITLE: Transgenic mice containing a disrupted p53 gene

DATE-ISSUED: October 29, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Donehower; Lawrence A.	Houston	TX	N/A	N/A
Bradley; Allan	Houston	TX	N/A	N/A
Butel; Janet S.	Houston	TX	N/A	N/A
Slagle; Betty	Bellaire	TX	N/A	N/A

APPL-NO: 08/ 278588

DATE FILED: July 21, 1994

PARENT-CASE:

This is a Continuation of application Ser. No. 07/816,740, filed Jan. 3, 1992, now abandoned which is a CIP of Ser. No. 07/637,563, filed Jan. 4, 1991, abandoned.

US-CL-CURRENT: 800/10, 424/9.1 , 800/18

ABSTRACT:

A desired non-human animal or an animal cell or human cell which contains a predefined, specific and desired alteration in at least one of its two p53 chromosomal alleles, such that at least one of these alleles contains a mutation which alters the expression of the allele, and the other of the alleles expresses either a normal p53 gene product, or comprises an identical or different p53 mutation.

4 Claims, 10 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 5

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Brief Summary Text - BSTX (49):
   Similar deletions in chromosome 17 have been identified in a wide variety of cancers including breast and lung cancers (Mackay, J. et al., Lancet ii:1384 (1988); James, C. D. et al., Canc. Res. 48:5546 (1988); Yakota, J. et al.,

Proc. Nat'l. Acad. Sci. (U.S.A.) 84:9252 (1987); Toguchida et al., Canc. Res. 48:3939 (1988)). In addition to p53 allele loss, Nigro et al. (Nature 342:705-708 (1989)) have demonstrated that the single remaining **p53 allele in a variety of human** tumors (brain, colon, breast, lung) undergo a point mutation which renders it tumorigenic. Fearon et al. (Cell 61:759-767 (1990)) have hypothesized that both point mutations and deletions in the p53 alleles may be required for a fully tumorigenic phenotype. These findings suggest that the p53 gene may have a role in many types of cancers.

Detailed Description Text - DETX (7):
    The invention also includes the embodiment wherein the tumor suppressing gene of the non-**human animal is a p53** gene.

Detailed Description Text - DETX (40):
    The present invention concerns a non-human animal or an animal (including human) cell in which one of the two naturally present copies of the **p53 gene of such non-human** animal or animal cell has been rendered non-functional through a mutation (such as a deletion, insertion, or substitution in the naturally occurring p53 gene sequence).

Detailed Description Text - DETX (43):
    In summary, there is now convincing evidence that the **human p53** gene is a tumor suppressor gene (Weinberg, R. A., Scientific Amer., Sept. 1988, pp 44-51). Like the RB protein, discussed above, p53 is a nuclear protein that forms a complex with SV40 large T antigen (DeCaprio, J. A. et al., Cell 54:275-283 (1988); Crawford, L. V., Int. Rev. Exper. Pathol. 25:1-50 (1983)). The binding of these two proteins by viral tumor antigens presumably inactivates them and contributes to transformation. p53 gene deletions have been noted in several mouse erythroleukemic cell lines, reminiscent of the RB gene deletions in retinoblastomas (Mowat et al., Nature 314:633-636 (1985); Chow et al., J. Virol. 61:2777-2781 (1987); Hicks, G. G. et al., J. Virol. 62:4752-4755 (1988)). Cell lines and tumors derived from human osteogenic sarcomas often contain gross rearrangements of the p53 gene, including deletions (Masuda, H., Proc. Nat'l. Acad. Sci. (U.S.A.) 84:7716-7719 (1987)). Allelic deletions in chromosome 17p (which contains the p53 gene) occur in over 75% of colorectal carcinomas (Baker, S. J. et al., Science 244:217-221 (1989)). In two tumors, the remaining non-deleted p53 allele was shown to contain mutants in highly conserved regions previously found to be mutated in murine p53 genes (Baker, S. J. et al., Science 244:217-221 (1989)). Loss of heterozygosity in chromosome 17p has been noted in a high percentage of individuals with small cell lung carcinoma (Yokoto, J. et al., Proc. Nat'l. Acad. Sci. (U.S.A.) 84:9252-9526 (1987); Harbour, J. W. et al., Science 241:353-356 (1988)). In the HL-60 human leukemic cell line, major deletions in the p53 gene and absence of the p53 protein have been noted (Wolf, D. et al., Proc. Nat'l. Acad. Sci. (U.S.A.) 82:790-794 (1985)). These results indicate that the absence of a functional p53 allele is highly correlated with some forms of cancer in humans and mice, strongly suggesting a tumor-suppressor role for p53. Finally, Finlay et al. (Cell 57:1083-1093 (1989)) have recently demonstrated that the wild-type mouse p53 gene suppresses transformation in vitro after cotransfection of rat embryo cells with E1a and activated ras,

indicating that the presence of the normal p53 gene acts negatively to block transformation.

Detailed Description Text - DETX (76):
    In contrast to the above-described methods, the present invention uses methods capable of producing subtle, precise, and predetermined mutations in the sequence of one of the two alleles of the **p53 gene of a human** or animal cell. Although the methods discussed below are capable of mutating both alleles of the cell's p53 gene, it is possible to readily identify (for example through the use of PCR (discussed below), or other methods) such dual mutational events. Since the frequency of such dual mutational events is the square of the frequency of a single mutational event, cells having mutations in both of their p53 alleles will be only a very small proportion of the total population of mutated cells.

Detailed Description Text - DETX (148):
    One especially preferred cell is a non-**human cell in which one of the natural p53 alleles has been replaced with a functional human p53** allele and the other of the natural p53 alleles has been mutated to a non-functional form. Alternatively, one may employ a non-**human cell in which the two natural p53** alleles have been replaced with a functional and a non-functional allele of the **human p53** gene.

Detailed Description Text - DETX (149):
    Such cells may be used, in accordance with the methods described above to assess the neoplastic potential of agents in cells containing the **human p53** allele. More preferably, such cells are used to produce non-human animals which do not contain any natural functional p53 alleles, but which contain only one functional **human p53** allele. Such non-human animals can be used to assess the tumorigenicity of an agent in a non-**human animal expressing the human p53** gene product.

Detailed Description Text - DETX (182):
    The transgenic cells and non-human animals of the present invention can be used to study **human gene regulation of the p53** gene. For example, such cells and animals can be used to investigate the interactions of the p53 gene with oncogenes or other tumor suppressor genes. Thus, they may be used to identify therapeutic agents which have the ability to impair or prevent neoplastic or tumorigenic development. Such agents have utility in the treatment and cure of . cancer in humans and animals.

Detailed Description Text - DETX (197):
    The gene targeting strategy utilized a murine p53 3.7 kb genomic construct flanked by an HSV TK gene and interrupted in exon 5 by a polyA - neo.sup.R gene driven by a pol II promoter. The 3.7 kb **fragment is derived from a genomic plasmid clone of murine p53** (Oren, M. et al., EMBO J. 2:1633-1639 (1983); Pinhasi et al. Molec. Cell. Biol. 4:2180-2186 (1984)). The DNA was cloned

from a liver cell of a normal Balb/c mouse. Following gene transfer into ES cells and G418/FIAU selection (as described above), two clones were identified by PCR and Southern analysis which have the expected altered p53 gene structure (FIG. 3). Three probes were used for this purpose. The first probe (neo probe) indicates the presence of the neomycin resistance determinant in the stem cells. The second and third probes (exon 1-2 and exon 5-10 probes) reveal that the wild type and mutant alleles are present in the two embryonic stem cells, but that the mutant allele is not present in the wild type (wt) cell. The fact that the three probes identify the same bands in the mutant embryonic stem cells indicates that, as expected, the neo determinant in these cells is linked to the p53 gene.

Detailed Description Text - DETX (204):

   As indicated above, a segment of the genomic sequences from the mouse **p53 gene (a 3.7-kb fragment** spanning exons 2 to 10 of the 11 exon gene) was obtained from plasmid pSVpcG3 (Pinhasi et al. Molec. Cell. Biol. 4:2180-2186 (1984)). As shown in FIG. 1, this sequence of DNA was modified in two ways. First, a neo marker gene driven by the MC1 promoter enhancer was designed to obtain high levels of expression in ES cells (Thomas, K. R. et al., Cell 51:503-512 (1988)). This sequence was inserted into the unique Bal 1 site in exon 5. This insertion provided a positive selectable marker (neo) for stable gene transfer into ES cells and disrupted the coding sequence of **p53,** producing an inactive allele following successful homologous recombination. The second alteration entailed the attachment of a herpes simplex virus thymidine **kinase** gene (HSV TK) to the 3' end of the gene-targeting construct (FIG. 2). This attachment provided the negative selection (using the HSV-TK-specific thymidine analogue FIAU (1-(2 deoxy, 2 fluoro, .beta.-D arabinofuranosyl)-5-iodouracil) against cells that have random integrations of the targeting construct (FIG. 2).

Detailed Description Text - DETX (238):

   Once the DNAs were obtained, their concentrations were determined by A.sub.260 determination on a UV spectrophotometer. 5-10 .mu.g of the DNAs were then cleaved with either BamHI or EcoR1 restriction endonucleases for several hours at 37.degree. C. The cut DNAs were loaded on a 0.8% agarose gel and electrophoresed at 100 V for approximately 3 hours on a horizontal submarine gel apparatus. The DNAs were then blotted to Zetaprobe (BioRad) nylon membranes and hybridized in 20% dextran sulfate-3.times.SSPE, both steps according to the protocol of Reed and Mann (Reed, K. C. et al., Nucleic Acids Res. 13:7207-7221 (1985)). The probe for hybridization was .sup.32 P-labelled according to the protocol of Feinberg and Vogelstein (Feinberg, A. et al., Anal. Biochem. 132:6-13 (1983)). Hybridization was permitted to continue overnight at 68.degree. C. and the filters were washed according to the protocol of Reed and Mann (Reed, K. C. et al., Nucleic Acids Res. 13:7207-7221 (1985))). The probe for FIG. 4 was a pol II-neo probe. The Pol II part of the probe hybridizes to a 4.0 kb fragment of the Pol II gene which is present in all mice. The Neo part of the probe hybridizes to a larger 6.7 kb **fragment present only in the p53** targeted germ line heterozygote mice.

US-PAT-NO: 5550023

DOCUMENT-IDENTIFIER: US 5550023 A

TITLE: Amplification of human MDM2 gene in human tumors

DATE-ISSUED: August 27, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kinzler; Kenneth W.	Baltimore	MD	N/A	N/A
Vogelstein; Bert	Baltimore	MD	N/A	N/A

APPL-NO: 08/ 245500

DATE FILED: May 18, 1994

PARENT-CASE:

   This application is a divisional of U.S. Ser. No. 08/044,619, filed Apr.
7, 1993, now U.S. Pat. No. 5,420,263 which is a continuation-in-part of U.S.
Ser. No. 07/903,103, filed Jun. 23, 1992, now U.S. Pat. No. 5,411,860 which
is a continuation-in-part of U.S. Ser. No. 07/867,840, filed Apr. 7, 1992,
now abandoned.

US-CL-CURRENT: 435/7.1, 435/7.2 , 435/7.21 , 435/7.23

ABSTRACT:

   A human gene has been discovered which is genetically altered in human tumor
cells. The genetic alteration is gene amplification and leads to a
corresponding increase in gene products. Detecting that the gene, designated
hMDM2, has become amplified or detecting increased expression of gene products
is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and
allows the cell to escape from p53-regulated growth. Methods of identifying
compounds which interfere with the binding of **human MDM2 protein to human p53**
protein involve measuring the amounts of the proteins bound, displaced, or
prevented from binding, in the presence of test compounds.

27 Claims, 24 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 17

---------- KWIC ---------

Abstract Text - ABTX (1):

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and allows the cell to escape from p53-regulated growth. Methods of identifying compounds which interfere with the binding of **human MDM2 protein to human p53** protein involve measuring the amounts of the proteins bound, displaced, or prevented from binding, in the presence of test compounds.

Brief Summary Text - BSTX (6):
  While there exists an enormous body of evidence linking **p53 gene mutations to human** tumorigenesis (Hollstein et at., 1991, Science 253:49-53) little is known about cellular regulators and mediators of p53 function.

Brief Summary Text - BSTX (7):
  Hinds et at. (Cell Growth & Differentiation, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated ras oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253:49-53. The transformed fibroblasts were found to produce elevated levels of **human p53** protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Brief Summary Text - BSTX (8):
  Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type **human p53** gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-496.

Brief Summary Text - BSTX (9):
  Hinds et al., supra also expressed **human p53** protein in transformed rat cells. When the expressed **human p53** was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified M.sub.r 90,000 protein was coimmunoprecipitated. This suggested that the rat M.sub.r 90,000 protein is in a complex with the **human p53** protein in the transformed rat cell line.

Brief Summary Text - BSTX (19):
  Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (22):
   Still another object of the invention is to provide polypeptides which
interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (24):
   It has now been discovered that hMDM2, a heretofore unknown human gene,
plays a role in human cancer. The hMDM2 gene has been cloned and the
recombinant derived hMDM2 protein shown to bind to **human p53** in vitro. hMDM2
has been found to be amplified in some neoplastic cells and the expression of
hMDM2-encoded products has been found to be correspondingly elevated in tumors
with amplification of this gene. The elevated levels of MDM2 appear to
sequester p53 and allow the cell to escape from p53-regulated growth.

Drawing Description Text - DRTX (7):
   FIG. 6 shows the determination of MDM2 and p53 domains of interaction. FIG.
5A and FIG. 5B. Random fragments of MDM2 were fused to sequences encoding the
lexA DNA binding domain and the resultant clones transfected into yeast
carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive
.beta.-galactosidase reporter). Yeast clones expressing .beta.-galactosidase
were identified by their blue color, and the MDM2 sequences in the lexA fusion
vector were determined. .beta.-galactosidase activity was observed independent
of p53 expression in A, but was dependent on p53 expression in B. The bottom 6
clones in B were generated by genetic engineering. FIG. 6C. Random **fragments
of p53** were fused to the sequence encoding the B42 acidic activation domain and
a hemagglutinin epitope tag; the resultant clones were transfected into yeast
carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and
pJK103. Yeast clones were identified as above, and all were found to be
MDM2dependent. The bottom three clones were generated by genetic engineering.

Drawing Description Text - DRTX (25):
   It has been found that amino acid residues 13-41 of **p53** (See SEQ ID NO:1)
are necessary for the interaction of MDM-2 and **p53**. However, additional
residues on either the amino or carboxy **terminal** side of the **peptide** appear
also to be required. Nine to 13 additional **p53** residues are sufficient to
achieve MDM2 binding, although less may be necessary. Since cells which
overexpress MDM2 escape from **p53**-regulated growth control in sarcomas, the use
of **p53-derived peptides to bind to excess MDM2 leads to reestablishment of
p53**-regulated growth control.

Drawing Description Text - DRTX (26):
   Suitable **p53-derived peptides** for administration are those which are
circular, linear, or derivitized to achieve better penetration of membranes,
for example. Other organic compounds which are modelled to achieve the same
three dimensional structure as the peptide of the invention can also be used.

Drawing Description Text - DRTX (27):

DNA encoding the MDM2-binding, **p53-derived peptide,** or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus, or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, inter alia encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of **peptide, the goal is to alleviate the sequestration of p53** by MDM2.

Drawing Description Text - DRTX (30):

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to **human p53**. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

Detailed Description Text - DETX (8):

To determine whether the hMDM2 protein could bind to **human p53** protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+(Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in FIG. 1 from nucleotide 312 to 2176. A 42 bp black beetle virus ribosome entry sequence (Dasmahapatra et at., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (E1-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Detailed Description Text - DETX (14):

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (FIG. 2, lanes 2 and 3). However, when in vitro translated **human p53** was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association in vitro (FIG. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et at., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (FIG. 2, lanes 8 and 9). When an in vitro translated mutant form of p53 (175.sup.his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

Detailed Description Text - DETX (40):

This assay was then applied to mapping the interaction domains of each

protein. Full length cDNA **fragments encoding MDM2 or p53** were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

Detailed Description Text - DETX (41):

METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K. W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D. M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector)and pJK103 (lexA-responsive .beta.-galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto galactose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent clones are diagrammed in FIG. 6A. The MDM2 sequences of the remaining 15 **p53-dependent clones coded for peptides** ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of FIG. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Detailed Description Text - DETX (42):

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-**terminal** to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three **p53** sequences shown in FIG. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated **fragments**. The resultant yeast colonies were examined for .beta.-galactosidase activity in situ. Of approximately 5000 clones containing MDM2 **fragments** fused to the lexA DNA binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of .beta.-galactosidase (about 5-fold less than the other fifteen clones) and .beta.-galactosidase expression was independent of **p53** expression (FIG. 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in

MDM2 (amino acids 230-301) (SEQ ID NO:3). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable .beta.-galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino **terminal** region of MDM2 (FIG. 6B). The .beta.-galactosidase activity of these clones was dependent on **p53** co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length **p53** contained MDM2 codons 1 to 118 (FIG. 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated **fragments** of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

Detailed Description Text - DETX (43):

In a converse set of experiments, yeast clones containing **fragments of p53** fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (FIG. 6C). The minimal overlap between these three **fragments** contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the **fragments** required 9-12 amino acids on either side of codons 13-41 for activity (FIG. 6C). To further test the idea that the amino **terminal** region of **p53** was required for MDM2 binding, we generated an additional yeast strain expressing the lexA-DNA binding domain fused to **p53** codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-**terminus of p53** were required for the interaction.

Detailed Description Text - DETX (44):

Sequence analysis showed that all **p53 and MDM2 fragments** noted in FIG. 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in FIG. 6 expressed the relevant proteins at similar levels, as shown by Western blotting (FIG. 7).

Claims Text - CLTX (12):

10. The method of claim 1 wherein the **p53 is human p53**.

Claims Text - CLTX (14):

contacting: a first polypeptide; a second polypeptide; and a compound to be tested for its capacity to interfere with binding of said first and second polypeptides to each other; wherein at least one of said first and said second polypeptides is a polypeptide which comprises less than all of the complete sequence of amino acids of **p53 or MDM2 proteins, wherein each of said**

polypeptides contain a sufficient portion f p53 or MDM2 proteins to bind to the other polypeptide; wherein when said first polypeptide is p53 or a polypeptide which comprises less than all of the complete sequence of amino acids of p53, then said second polypeptide is MDM2 or a polypeptide which comprises less than all of the complete sequence of amino acids of MDM2; and when said first polypeptide is MDM2 or a polypeptide which comprises less than all of the complete sequence of amino acids of MDM2, then said second polypeptide is p53 or a polypeptide which comprises less than all of the complete sequence of amino acids of p53; and

Claims Text - CLTX (20):
   16. The method of claim 11 wherein said polypeptide which comprises less than all of the complete sequence of amino acids of p53 comprises amino acids 1-41 of **human p53** as shown in SEQ ID NO:1.

Claims Text - CLTX (21):
   17. The method of claim 11 wherein said polypeptide which comprises less than all of the complete sequence of amino acids of p53 comprises amino acids 13-57 of **human p53** as shown in SEQ ID NO:1.

Claims Text - CLTX (22):
   18. The method of claim 11 wherein said polypeptide which comprises less than all of the complete sequence of amino acids of p53 comprises amino acids 1-50 of **human p53** as shown in SEQ ID NO:1.

Claims Text - CLTX (25):
   21. The method of claim 11 wherein the **p53 is human p53**.

Claims Text - CLTX (27):
   23. The method of claim 22 wherein said fusion polypeptide comprises amino acids 1-41 of **human p53** as shown in SEQ ID NO:1.

Claims Text - CLTX (28):
   24. The method of claim 22 wherein said fusion polypeptide comprises amino acids 13-57 of **human p53** as shown in SEQ ID NO:1.

Claims Text - CLTX (29):
   25. The method of claim 22 wherein said fusion polypeptide comprises amino acids 1-50 of **human p53** as shown in SEQ ID NO:1.

Claims Text - CLTX (31):
   27. A method of determining the quantity of **human p53 which binds to human** MDM2, or of **human MDM2 which binds to human p53,** said method comprising:

Claims Text - CLTX (32):
    contacting: a first protein and a second protein, wherein the first protein
is human MDM2 and the second protein is **human p53 or the first protein is human**
p53 and the second protein is human MDM2; and

Other Reference Publication - OREF (2):
    Hinds, et al., "Mutant **p53 DNA Clones From Human** Colon Carcinomas Cooperate
With Ras in Transforming Primary Rat Cells: A Comparison of the Hot Spot Mutant
Phenotypes," Cell Growth & Differential, 1:561-580 (1990).

Other Reference Publication - OREF (5):
    Oliner, et al., "Amplification of a Gene Encoding a **p53-Associated Protein**
in Human Sarcomas", Nature, 358:80-83 (1992).

Other Reference Publication - OREF (7):
    Leach, et al., "**p53 Mutation and MDMS Amplification in Human** Soft Tissue
Sarcomas", Cancer Research 53:2231-2234 (1993).

US-PAT-NO: 5532348

DOCUMENT-IDENTIFIER: US 5532348 A

TITLE: E6 associated protein and methods of use thereof

DATE-ISSUED: July 2, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Huibregtse; Jon M.	Brighton	MA	N/A	N/A
Scheffner; Martin	Walldorf	N/A	N/A	DE
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APPL-NO: 08/ 100692

DATE FILED: July 30, 1993

US-CL-CURRENT: 536/23.5, 435/235.1

ABSTRACT:

   The present invention provides compositions of isolated and purified E6
Associated Protein and fragments thereof. Also provided are nucleic acid
constructs encoding E6 Associated Protein. These compositions may be employed
to identify compounds which inhibit binding of high risk HPV E6 to p53. The
compositions of the present invention may also be used in methods to detect the
presence of high risk HPV in biological samples.

5 Claims, 21 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 12

---------- KWIC ---------

Brief Summary Text - BSTX (3):
   Malignant transformation of cells has been linked to expression of proteins
encoded by oncogenes (Huang et al., Cell, 39:79-87 (1984)). Cells may also be
transformed by suppression of growth- and replication-inhibiting factors.
Suppression of these inhibiting factors may allow unrestrained cell replication
and malignant transformation. Lane and Benchimol, Genes and Devel., 4:1-8
(1990). **Human protein p53** is one such inhibiting factor.

Brief Summary Text - BSTX (4):
   Many lines of evidence point to the importance of protein **p53 in human**

carcinogenesis. Mutations within the p53 gene are the most frequent genetic aberration thus far associated with human cancer (Vogelstein, Nature, 348:681-682 (1990)) and individuals with germ line p53 mutation have an elevated risk of developing cancer (Malkin et al., Science, 250:1233-1238 (1990); Srivastava et al., Nature, 348:747-749 (1990)). The mutations identified in cancers are generally point mutations which fall within evolutionarily conserved domains and most of these mutated alleles have transforming activity in various cell culture assays (reviewed in Lane and Benchimol, supra and Levine et al., Nature, 351:453-456 (1991)).

Brief Summary Text - BSTX (15):

Methods for detecting human papillomavirus associated with a high risk of malignancy in a biological sample are also provided. The methods generally comprise contacting the sample with a composition comprising mammalian tumor suppressor protein p53 and purified E6 Associated Protein under conditions conducive to complex formation, detecting formation of complexes between E6 and **p53 therein, and determining the presence of human** papillomavirus infection associated with a high risk of malignancy. The present invention also provides methods for identifying compounds which inhibit binding of E6 to p53. The methods generally comprise contacting compounds of interest with isolated and purified E6-AP to form a mixture, adding the mixture to a composition comprising E6 and p53, but not containing E6 Associated Protein, and detecting formation of complexes of E6 and p53.

Detailed Description Text - DETX (7):

These E6-AP peptides of the present invention may be employed to inhibit binding of high risk E6 proteins to p53. By "high risk E6 proteins", it is meant E6 proteins produced by papillomaviruses associated with a high risk of malignant degeneration following cellular infection. Such "high risk E6 proteins" include E6 proteins from human papillomaviruses 16 or 18. **Peptides of the present invention may be synthesized which will bind E6, p53,** or both. This may block the E6 mediated degradation of p53. Further, E6-AP proteins having mutations in regions necessary for p53 degradation by ubiquitination may be synthesized to block p53 ubiquitination.

Detailed Description Text - DETX (65):

E6-AP can stably associate with high risk HPV E6 proteins in the absence of **p53**. The largest segment of E6-AP fused to GST consisted of the C-**terminal** 653 amino acids (amino acids 212-865 of SEQ ID NO:1). This 75 kDa **fragment** of E6-AP contains all of the sequences necessary to direct association of E6 with **p53** and induce degradation of **p53** by ubiquitination. Equal amounts (approximately 0.1 .mu.g) of different GST-E6-AP **fragment** fusion proteins were assayed for the ability to associate with HPV16 or HPV11 E6 proteins by mixing the GST fusion proteins, immobilized on glutathione-Sepharose, with in vitro-translated .sup.35 S-labeled E6 proteins. Wheat germ extract was used for translation of E6 proteins. The level of binding considered non-specific was that amount of E6 protein that bound to GST lacking E6-AP protein sequences. FIG. 8A illustrates a schematic representation of the regions of E6-AP assayed for E6 association. FIG. 8B illustrates binding of the fusion proteins to labeled E6. The 75 kDa form of the E6-AP and the amino-**terminal**

p rtion (amino acids 213 to 489 of SEQ ID NO:1) of the this region bound specifically to HPV16 E6. The carboxy-**terminal portion** of E6-AP (amino acids 544 to 865 of SEQ ID NO:1) did not bind to HPV16 E6. Binding assays of additional fusion proteins containing amino acid sequences of the amino-**terminal** region demonstrated that the E6 binding domain is between amino acids 371 and 440 of SEQ ID NO:1 (also referred to as SEQ ID NO: 2). This E6-AP **fragment** is encoded by SEQ ID NO:5. These fusion proteins bound approximately 50% of the input .sup.35 S-labeled HPV16 E6. None of the E6-AP fusion proteins bound HPV11 E6 above the background level.

Detailed Description Text - DETX (74):

p53 ubiquitination assays were performed by combining 2 .mu.l of .sup.35 S-labeled wheat germ extract-translated **human wild-type p53** with 10 .mu.l of either a mock wheat germ extract translation reaction mixture or a wheat germ extract translation mixture programmed with HPV16 E6 mRNA and with 10 .mu.l of the DEAE fraction from either uninfected S19 cells or baculovirus-infected S19 cells. Additionally, each reaction mixture contained 24 .mu.l of T.sub.25 N.sub.50, 2 .mu.l of 2 mg/ml ubiquitin (Sigma), and 2 .mu.l of 40 mM ATP-.gamma.-S (total volume, 50 .mu.l). The mixtures were incubated at room temperature for 4 hours and then analyzed by SDS-PAGE and fluorography.

Other Reference Publication - OREF (1):

AA. Huibregtse, et al., "Cloning and Expression of the cDNA for E6-AP, a Protein That Mediates the Interaction of the **Human Papillomavirus E6 Oncoprotein with p53**" Mol. Cell. Biol. 13(2):775-84 (Feb. 1993).

Other Reference Publication - OREF (19):

Huibregtse et al., "A Cellular Protein Mediates Association of **p53 with the E6 Oncoprotein of Human** Papillomavirus Types 16 or 18", EMBO J., 10:4129-4135 (1991).

Other Reference Publication - OREF (21):

Scheffner et al., "The state of the **p53 and retinoblastoma genes in human** cervical carcinoma cell lines", Proc. Natl. Acad. Sci. USA, 88:5523-5527 (1991).

Other Reference Publication - OREF (26):

Oliner et al., "Amplification of a Gene Encoding a **p53-associated Protein in Human** Sarcomas", Nature, 358:80-83 (Jul. 2, 1992).

US-PAT-NO: 5532220

DOCUMENT-IDENTIFIER: US 5532220 A
See image for Certificate of Correction

TITLE: Genetic mechanisms of tumor suppression

DATE-ISSUED: July 2, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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APPL-NO: 08/ 337851

DATE FILED: November 14, 1994

PARENT-CASE:

   This application is a continuation of application Ser. No. 07/947,359,
filed Sep. 18, 1992 now abandoned; which is a divisional of U.S. Ser. No.
07/573,405 filed Aug. 24, 1990 now abandoned; which is a continuation-in-part
of U.S. Ser. No. 07/091,547 filed Aug. 31, 1987 (patent issued: U.S. Pat.
No. 5,011,773); and a continuation-in-part of Ser. No. 07/108,748 filed Oct.
15, 1987 now abandoned; and a continuation-in-part of Ser. No. 07/265,829
filed Oct. 31, 1988 (abandoned); and a continuation-in-part of Ser. No.
07/533,892 filed Jul. 16, 1990, and a continuation-in-part of U.S. Ser. No.
07/553,905 filed Jul. 16, 1990 now abandoned.

US-CL-CURRENT: 514/44, 424/93.21 , 435/320.1 , 435/456

ABSTRACT:

   A method for utilizing p53 cDNA, and p53 gene products for the suppression
of the neoplastic phenotype.

6 Claims, 16 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 9

---------- KWIC ---------

Drawing Description Text - DRTX (5):
   FIG. 2A is a chromatogram depicting expression of **human p53** proteins in
virus-producing cell lines;

Drawing Description Text - DRTX (6):

FIG. 2B is a chromatogram depicting half-life determination of **human p53** in virus-producing lines by pulse-chase labeling experiments;

Drawing Description Text - DRTX (7):

FIG. 3A, 3B and 3C depict the expression of **human p53** proteins in virus-infected Saos-2 cells;

Detailed Description Text - DETX (2):

In FIG. 1A, three **human p53** cDNAs are diagrammed. The sequence reported by Lam and Crawford, Mol. Cell. Biol. 6, 1379-1385 (1986), here labelled as p53L, was derived by sequencing clones from human fetal liver cDNA and genomic libraries, and is considered to be wild-type. p53B is a cDNA clone derived from fetal brain RNA by the RT-PCR method which resulted in cloning of wild type **p53** (p53B) cDNA as follows: about 15 .mu.g of fetal brain RNA were mixed with 1.5 .mu.g of oligo (dT) primer and 60 units of avian myeloblastosis virus reverse transcriptase in cDNA buffer (50 mM Tris-HCl, pH 8.0, 80 mM KCl, 5 mM MgCl2, 1 mM each dATP, dGTP, dTTP, and dCTP). The reaction mixture was incubated for 90 min at 42.degree. C. After reaction, RNA was degraded with 0.5N NaOH, and single-stranded cDNA was precipitated with ethanol. PCR amplification was carried out with one-tenth of the cDNA, 100 ng of each oligonucleotide primer (5'-TGCAAGCTTTCCACGACGGTGACACGCT-3' and 5'-AGTGCAGGCCA-ACTTGTTCAGTGGA-3'), and 5U of Taq polymerase in PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgC12, and 0.001% gelatin) for 40 cycles in a programmable heat block (Ericomp, San Diego, Calif.). Each cycle included denaturation at 93.degree. C. for 1 minute, reannealing at 62.degree. C. for 80 seconds, and primer extention at 72.degree. C. for 3 minutes. PCR products were extracted with phenol and precipitated with ethanol. The precipitate was dissolved in H.sub.2 O and digested with restriction enzymes (Hind III and Sma 1). The p53cDNA **fragment** was subcloned into virus vector to form Vp53B-Neo. Subcloned p53B was sequenced by using the dideoxy chain **termination** method (F. Sanger, S. Nicklen, A. R. Coulsen, Proc. Natl. Acad. Sci, U.S.A. 74, 5463 (1977)).

Detailed Description Text - DETX (5):

FIG. 2A is a chromatogram depicting Murine PA 12 cells (Lane m1), human WERI-Rb27 retinoblastoma cells (Lane m2), and Vp53En-, Vp53BN-, or Vp53BH-producing PA 12 cells which were metabolically labelled with .sup.35 -methionine. Cell lysates were immunoprecipitated with anti-p53 antibody PAb421 utilizing conventional methods. Immunoprecipitates were separated by 8.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and autoradiographed. marker lanes m1 and m2 show endogenous murine p53 (Mp53) and both polymorphic forms of **human p53** (Hp53). Human p53B (filled arrow) and p53E (open arrow) proteins in mouse cells are indicated.

Detailed Description Text - DETX (6):

FIG. 2B depicts half-life determination of **human p53** in virus-producing

lines by pulse-chase labeling experiments. PA12 cells expressing p53E (panel a) or p53B (panels b & c, representing two independent clones) were labelled with 0.25 mCi/ml .sup.35 S-methionine for 60 minutes, and chased with a 1000-fold molar excess of unlabeled methionine. At the indicated times, cells were harvested for immunoprecipitation of p53 protein with PAb421 as described above. The half-life of p53B was 20-30 minutes whereas that of p53E was 4-5 hours. Marker lanes m1 and m2, and filled and open arrows, were as in FIG. 2A.

Detailed Description Text - DETX (7):

   FIG. 3 is a chromatogram depicting expression of **human p53** proteins in virus-infected Saos-2 cells. Saos-2 cells (lanes 1 and 7) were infected with Vp53E-Neo, Vp53B-Neo, or Vp53B-Hygro to generate p53EN (lanes 2-6), p53BN (lanes 8-10), or p53BH (lanes 11 and 12) clones, respectively. Saos-2 cells were also doubly infected with Vp53E-Neo and Vp53B-Hygro to generate p53EN-BH clones (lanes 13-15). Randomly-selected clones, and WERI-Rb27 cells (lanes M), were labeled with .sup.35 S-methionine and immunoprecipitated with PAb421 as described with regard to FIG. 2A. p53B (filled arrows) and p53E (open arrows) are indicated.

Detailed Description Text - DETX (14):

   p53 (FIG. 9) was originally identified as a mammalian cellular protein that binds to SV40T antigen, a property that is also shared by RB protein. Deletions or rearrangements of the murine or **human gene encoding p53** were found in Friend virus-induced murine erythroleukemias, and in human osteosarcomas, lung carcinomas, lymphomas and leukemias. On the other hand, many human breast, lung and colon carcinomas expressed high levels of aberrant p53 species with markedly prolonged half-lives due to certain point mutations in the p53 gene (Genes Devel. 4, 1-8 (1990)). These observations suggest that mutation of **p53 contributes to human** oncogenesis. p53 was originally considered to be an oncogene because it was known that it could transform primary rat embryo fibroblasts in concert with an activated ras gene. However, the observation of p53 deletions, and point mutations scattered over several exons, also suggested that p53 might be a tumor suppressor gene, i.e., a gene that was inactivated by mutation. Indeed, Finlay et al. and Eliyahu et al. (Cell 57, 1083-1093 (1989)) Proc. Natl. Acad. Sci U.S.A. 86, 8763-8767 (1989)) found that cotransfection of murine wild-type p53 DNA could reduce the transformation efficiency of transfected oncogenes in rat embryo fibroblasts, whereas mutated p53 DNA enhanced such transformation. The dominant transforming effect was presumed to be due to a "dominant negative" activity of mutated p53 protein that somehow blocked the growth-restricting function of wild-type p53 protein in cells. This model suggested that the relative quantity of mutated to wild-type p53 could determine the transformed phenotype, but gene dosage could not be tightly controlled in these transfection studies.

Detailed Description Text - DETX (15):

   Because of such technical questions, as well as the possibility of species-specific differences in p53 function and the uncertain relevance of transformed animal cells to human neoplasia, it was determined that the biological properties of **p53 in the human** system should be reassessed. It was recognized that an ideal host cell for these studies would allow the

experimental manipulation of single copies of mutated or wild-type p53 alleles. However, most cultured human cells contain endogenous and possibly mutated p53 alleles that are not accessible to external control. The human osteosarcoma cell line Saos-2 was therefore chosen because it has no endogenous p53 due to complete deletion of its gene. Recombinant retroviruses derived from Moloney murine leukemia virus (Mo-MuLV) were used to introduce mutated and/or wild-type p53 under LTR promoter control. Cell clones isolated after infection and selection carried only a single integrated provirus of each type, and multiple clones were analyzed to exclude positional effects. A comprehensive assessment of biological properties of these clones included morphology, growth rates and saturation density in culture, colony formation in soft agar, and tumorigenicity in nude mice.

Detailed Description Text - DETX (17):

As a reference standard for **human wild-type p53,** the genomic DNA sequence of Lamb and Crawford (Mol. Cell. Biol. 6, 1379-1385 (1986)) was used. Potentially wild-type **p53** cDNA was isolated from fetal brain RNA by the method of RT-PCR, and was cloned into plasmid. In cloning of wild type **p53** (p53B) cDNA about 5 .mu.g of fetal brain RNA were mixed with 1.5 .mu.g of oligo(dT) primer and 60 units of avian myeloblastosis virus reverse transcriptase in cDNA buffer (50 mM Tris-HCl, pH 8.0, 80 mM KCl, 5 mM MgCl.sub.2, 1 mM each dATP, dGTP, dTTP, and dCTP). The reaction mixture was incubated for 90 min at 42.degree. C. After reaction, RNA was degraded with 0.5N NaOH, and single-stranded cDNA was precipitated with ethanol. PCR amplification was carried out with one-tenth of the cDNA, 100 ng of each oligonucleotide primer (5'-TGCAAGCTTTCCACGACGGTGACACGCT-3' and 5-AGTGCAGGCCA-ACTTGTTCAGTGGA-3'), and 5 U of Taq polymerase in PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl.sub.2, and 0.001% gelatin) for 40 cycles in a programmable heat block (Ericomp, San Diego, Calif.). Each cycle included denaturation at 93.degree. C. for 1 minute, reannealing at 62.degree. for 80 seconds, and primer extension at 72.degree. C. for 3 minutes. PCR products were extracted with phenol and precipitated with ethanol. The precipitate was dissolved in H.sub.2 O and digested with restriction enzymes (Hind III and Sma 1). The **p53 cDNA fragment** was subcloned into virus vector to form Vp53B-Neo. Subcloned p53B was sequenced by using the dideoxy chain **termination** method (Proc. Natl. Acad. Sci. U.S.A. 74, 5463 (1977)).

Detailed Description Text - DETX (19):

p53E and p53B were then inserted into a Mo-MuLV-based retroviral vector containing neo as a selectable marker gene to form Vp53E-Neo and Vp53B-Neo viral genomes, respectively (FIG. 1B). In addition, to facilitate double replacement, Vp53B-Hygro was made by inserting p53B into a similar vector containing the gene which is known to confer resistance to hygromycin. Stocks of Vp53E-Neo, Vp53B-Neo and Vp53B-Hygro viruses were produced, utilizing conventional methods, with titers of about 1.times.10.sup.5, 2.times.10.sup.4, and 1.times.10.sup.5, respectively. Expression of p53 proteins from the viruses was initially assessed in the murine NIH3T3-derived packaging line, PA12, that was used for virus production. Mutated and wild-type **human p53** proteins were detected in their respective virus-producing cells, with the expected difference in migration by SDS-PAGE (FIG. 2A). Because spontaneous

mutation of p53 may occur frequently in cultured cells, two additional biochemical properties of these p53 proteins were examined. These were their cellular half-lives, and their ability to bind to T antigen. p53B protein had a half-life of 20-30 minutes compared to 4 to 5 hours for p53E protein (FIG. 2B), consistent with published reports on the half-lives of wild-type and mutated p53 proteins. When virus-producing cells were transfected with a plasmid expressing large amounts of SV40T antigen, and lysates were immunoprecipitated with anti-p53 or anti-T antibodies, T antigen was coprecipitated with p53B but not p53E protein, indicating that only p53B protein could bind to T. These results together suggested that p53B-containing viruses expressed wild-type, and that p53E containing virus expressed mutated p53.

Detailed Description Text - DETX (30):

Introduction of wild-type **p53 in human osteosarcoma cells lacking p53** expression clearly suppressed their neoplastic phenotype, indicating that p53 can function as a tumor suppressor gene in this system. Conversely, insertion of mutated p53 into these cells augmented one aspect of their growth in culture (saturation density), thereby showing that mutated p53 retains a limited function, albeit one that was overridden by wild-type p53. These results are broadly consistent with those of other investigators who have addressed the influence of wild-type murine p53 on oncogene-mediated transformation of primary rat embryo fibroblasts. In these studies, cotransfection of plasmid DNA containing the wild-type p53 gene markedly reduced the transformation efficiency of several activated oncogenes, either singly or in combinations such as ras+myc or ras+E1A. Mutated p53 did not have this suppressive effect, and instead modestly boosted transformation efficiency. Wild-type p53 was also effective in reducing transformation by mutated p53 in concert with other oncogenes, suggesting "dominance" of the wild-type suppression function. Colonies recovered after transfection with wild-type p53 DNA either failed to express exogenous p53, or expressed only mutated p53.

Detailed Description Text - DETX (33):

It is known that murine p53 genes cloned from many cultured cell lines have point mutations that cluster in five conserved regions. This class of mutation was responsible for the initial impression that **p53 was a dominant oncogene, because such p53 DNA fragments** or constructs were active in promoting transformation of rodent cells in a variety of assays. Furthermore, protein products of mutated p53 genes have common antigenic and biochemical characteristics that differ from wild-type p53 protein, including a prolonged half-life that results in abnormally high cellular p53 protein levels. These features are quite reminiscent of other dominant oncogenes like myc and ras. On the other hand, gross deletions or rearrangements of the p53 gene, incompatible with expression of a gene product have been found in Friend-virus induced murine erythroleukemias, (Nature 314, 633-636 (1985)). Such mutations are considered to be characteristic of so-called tumor suppressor genes, and serve to inactivate their normal function. To explain how both kinds of mutation could impart the same oncogenic phenotype, it was proposed that wild-type p53 indeed functioned to suppress tumor formation, and that the many known point mutations of p53 actually served to inactivate this function. To explain the dominant transforming activity of mutated p53 genes in primary

cells, it was necessary to hypothesize that mutated p53 protein somehow inactivated endogenous, wild-type p53 protein. This "dominant negative" effect might occur by inhibitory interactions between mutated and wild-type proteins, (Nature, 329, 219-222 (1987)). Further interpretation of these studies was limited by the technical drawbacks of transfection, and by the uncertain role of endogenous p53 in primary cells.

Detailed Description Text - DETX (43):

Mutations of the gene encoding p53, a 53 kD cellular protein, are found frequently in human tumor cells, suggesting a crucial role for this gene in human oncogenesis. In order to model the stepwise mutation or loss of both **p53 alleles during human** oncogenesis, a human osteosarcoma cell line, Saos-2 was utilized that lacked endogenous p53 due to complete deletion of the gene. Single copies of exogenous p53 genes were then introduced by infecting cells with recombinant retroviruses containing either wild-type or point-mutated versions of the p53 cDNA sequence. It was found that 1) expression of wild-type p53 suppresses the neoplastic phenotype of Saos-2 cells; 2) expression of mutated p53 confers a limited growth advantage to cells in the absence of wild-type p53; and 3) wild-type p53 is phenotypically dominant to mutated p53 in a two-allele configuration. These results indicate that, as with the retinoblastoma gene, mutation of both alleles of the p53 gene is essential for its role in oncogenesis.

Other Reference Publication - OREF (3):

Banks et al., "Isolation of **human-p53**-specific monoclonal antibodies and their use in the studies of **human p53** expression." Eur. J. Biochem. 159:529-534 (1986).

Other Reference Publication - OREF (18):

Harlow et al., "Molecular Cloning and In Vitro Expression of a cDNA Clone for **Human Cellular Tumor Antigen p53.**" Molecular and Cellular Biol. 5(7):1601-1610 (1985).

Other Reference Publication - OREF (20):

Harris et al., "Molecular Basis for Heterogeneity of the **Human p53** Protein." Molecular and Cellular Biol. 6(12) 4650-4656 (1986).

Other Reference Publication - OREF (30):

Lamb et al., "Characterization of **Human p53** Gene." Molecular and Cellular Biol. 6(5):1379-1385 (1986).

Other Reference Publication - OREF (42):

Matlashewski et al., "Isolation and characterization of a **human p53 cDNA clone: expression of the human p53** gene." EMBO J. 3(13):3257-3262 (1984).

Other Reference Publication - OREF (44):
   Mercer et al., "Negative growth regulation in a glioblastoma tumor cell line
that conditionally expresses **human wild-type p53.**" Proc. Natl. Acad. Scie USA
87:6166-6170 (1990).

Other Reference Publication - OREF (52):
   Nigro, Janice, "Mutations in the **p53 gene occur in diverse human** tumour
types." Nature 342:705-708 (1989).

Other Reference Publication - OREF (54):
   Prokocimer et al., "Expression of **p53 in human** leukemia and lymphoma." Blood
68(1):113-118 (1986).

Other Reference Publication - OREF (61):
   Soussi et al., "Cloning and characterization of a cDNA from Xenopus laevis
coding for a protein homologous to **human and murine p53.**" Oncogene 1(1):71-78
(1987).

Other Reference Publication - OREF (65):
   Wills et al., "Development and Characterization of Recombinant Adenoviruses
Encoding **Human p53 for Gene Therapy of Cancer." Human** Gene Therapy 5:1079-1088
(1994).

Other Reference Publication - OREF (66):
   Wolf et al., "In Vitro Expression of **Human p53** cDNA Clones and
Characterization of the Cloned **Human p53** Gene." Molecular and Cellular Biol.
5(8):1887-1893 (1985).

Other Reference Publication - OREF (67):
   Zakut-Houri et al., "**Human p53** cellular tumor antigen: cDNA sequence and
expression in COS cells." The EMBO Journal 4(5):1251-1255 (1985).

US-PAT-NO: 5420263

DOCUMENT-IDENTIFIER: US 5420263 A
See image for Certificate of Correction

TITLE: Amplification of human MDM2 gene in human tumors

DATE-ISSUED: May 30, 1995

INVENTOR-INFORMATION:

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Vogelstein; Bert	Baltimore	MD	N/A	N/A

APPL-NO: 08/ 044619

DATE FILED: April 7, 1993

PARENT-CASE:

   This application is a continuation-in-part of U.S. Ser. No. 07/903,103,
filed Jun. 23, 1992, which is a continuation-in-part of U.S. Ser. No.
07/867,840, filed Apr. 7, 1992, now abandoned.

US-CL-CURRENT: 536/23.1, 435/6 , 536/23.2 , 536/24.3

ABSTRACT:

   A human gene has been discovered which is genetically altered in human tumor
cells. The genetic alteration is gene amplification and leads to a
corresponding increase in gene products. Detecting that the gene, designated
hMDM2, has become amplified or detecting increased expression of gene products
is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and
allows the cell to escape from p53-regulated growth.

17 Claims, 13 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 16

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Abstract Text - ABTX (1):
   A human gene has been discovered which is genetically altered in human tumor
cells. The genetic alteration is gene amplification and leads to a

corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to **human p53** and allows the cell to escape from p53-regulated growth.

Brief Summary Text - BSTX (6):
   While there exists an enormous body of evidence linking **p53 gene mutations to human** tumorigenesis (Hollstein et al., 1991, Science 253:49-53) little is known about cellular regulators and mediators of p53 function.

Brief Summary Text - BSTX (7):
   Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that p53 eDNA clones, containing a point mutation at amino acid residue 143, 175,273 or 281, cooperated with the activated ras oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253:49-53. The transformed fibroblasts were found to produce elevated levels of **human p53** protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Brief Summary Text - BSTX (8):
   Mutant p53 proteins with mutations at residue 143 or 175 form an oligomefic protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 routants do not detectably bind hsc70, and are poorer at producing transformed loci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type **human p53** gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

Brief Summary Text - BSTX (9):
   Hinds et al., supra also expressed **human p53** protein in transformed rat cells. When the expressed **human p53** was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53 an unidentified M.sub.r 90,000 protein was coimmunoprecipitated. This suggested that the rat M.sub.r 90,000 protein is in a complex with the **human p53** protein in the transformed rat cell line.

Brief Summary Text - BSTX (19):
   Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (22):
   Still another object of the invention is to provide polypeptides which interfere with the binding of **human MDM2 to human p53**.

Brief Summary Text - BSTX (23):

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to **human p53** in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

Drawing Description Text - DRTX (7):

FIG. 6 shows the determination of MDM2 and p53 domains of interaction. FIG. 5A and FIG. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive .beta.-galactosidase reporter). Yeast clones expressing .beta.-galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined. .beta.-galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. FIG. 6C. Random **fragments of p53** were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Detailed Description Text - DETX (13):

It has been found that amino acid residues 13-41 of **p53** (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and **p53**. However, additional residues on either the amino or carboxy **terminal** side of the **peptide** appear also to be required. Nine to 13 additional **p53** residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from **p53**-regulated growth control in sarcomas, the use of **p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53**-regulated growth control.

Detailed Description Text - DETX (14):

Suitable **p53-derived peptides** for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

Detailed Description Text - DETX (15):

DNA encoding the MDM2-binding, **p53-derived peptide,** or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus, or plasmid vector, which has the DNA elements necessary

for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, inter alia encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of **peptide, the goal is to alleviate the sequestration of p53** by MDM2.

Detailed Description Text - DETX (18):

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to **human p53**. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

Detailed Description Text - DETX (27):

To determine whether the hMDM2 protein could bind to **human p53** protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in FIG. 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Detailed Description Text - DETX (33):

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (FIG. 2, lanes 2 and 3). However, when in vitro translated **human p53** was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association in vitro (FIG. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (FIG. 2, lanes 8 and 9). When an in vitro translated mutant form of p53 (175.sup.his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

Detailed Description Text - DETX (59):

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA **fragments encoding MDM2 or p53** were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

Detailed Description Text - DETX (60):

METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K. W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D. M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector)and pJK103 (lexA-responsive .beta.-galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gat-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galaclose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent clones are diagrammed in FIG. 6A. The MDM2 sequences of the remaining 15 **p53-dependent clones coded for peptides** ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of FIG. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Detailed Description Text - DETX (61):

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-**terminal** to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three **p53** sequences shown in FIG. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated **fragments**.

Detailed Description Text - DETX (62):

The resultant yeast colonies were examined for .beta.-galactosidase activity in situ. Of approximately 5000 clones containing MDM2 **fragments** fused to the lexA DNA binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of .beta.-galactosidase (about 5-fold less than the other fifteen clones) and .beta.-galactosidase expression was independent of **p53** expression (FIG. 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This

acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable .beta.-galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino **terminal** region of MDM2 (FIG. 6B). The .beta.-galactosidase activity of these clones was dependent on **p53** co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length **p53** contained MDM2 codons 1 to 118 (FIG. 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated **fragments** of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

Detailed Description Text - DETX (63):

In a converse set of experiments, yeast clones containing **fragments of p53** fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (FIG. 2C). The minimal overlap between these three **fragments** contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the **fragments** required 9-12 amino acids on either side of codons 13-41 for activity (FIG. 6C). To further test the idea that the amino **terminal** region of **p53** was required for MDM2 binding, we generated an additional yeast strain expressing the lexA-DNA binding domain fused to **p53** codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-**terminus of p53** were required for the interaction.

Detailed Description Text - DETX (64):

Sequence analysis showed that all **p53 and MDM2 fragments** noted in FIG. 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in FIG. 6 expressed the relevant proteins at similar levels, as shown by Western blotting (FIG. 7).

Other Reference Publication - OREF (3):

Hinds, et al., "Mutant **p53 DNA Clones From Human** Colon Carcinomas Cooperate With Ras in Transforming Primary Rat Dells: A Comparison of the Hot Spot Mutant Phenotypes", Cell Growth & Differentiation, 1:561-580 (1990).

Other Reference Publication - OREF (6):

Oliner, et al., "Amplification of a Gene Encoding a **p53-Associated Protein in Human** Sarcomas", Nature, 358:80-83 (1992).

Other Reference Publication - OREF (8):

Leach, et al., "**p53 Mutation and MDMS Amplification in Human** Soft Tissue Sarcomas", Cancer Research 53:2231-2234 (1993).

US-PAT-NO: 5362623

DOCUMENT-IDENTIFIER: US 5362623 A
See image for Certificate of Correction

TITLE: Sequence specific DNA binding by p53

DATE-ISSUED: November 8, 1994

INVENTOR-INFORMATION:
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Kinzler; Kenneth W.	Baltimore	MD	N/A	N/A
Sherman; Michael I.	Glen Ridge	NJ	N/A	N/A

APPL-NO: 07/ 860758

DATE FILED: March 31, 1992

PARENT-CASE:

   This application is a continuation-in-part of U.S. Ser. No. 07/715,182
filed Jun. 14, 1991.

US-CL-CURRENT: 435/6, 435/320.1 , 536/24.1 , 536/24.31

ABSTRACT:

   Specific sequences in the human genome are the sites of strong binding of
wild-type p53 protein, but not mutant forms of the protein. These sequences
are used diagnostically to detect cells in which the amount of wild-type p53 is
diminished. The sequences can also be used to screen for agents which correct
for loss of wild-type p53 to DNA in cancer cells.

12 Claims, 31 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 27

---------- KWIC ---------

Brief Summary Text - BSTX (9):
   It is yet another object of the invention to provide a double-stranded DNA
fragment which contains a p53-specific DNA binding site.

Brief Summary Text - BSTX (13):

These and other objects of the invention are provided to one or more of the embodiments described below. In one embodiment a method is provided for detecting the presence of wild-type p53 protein in a cell, comprising the steps of: contacting a **p53-specific-binding DNA fragment** with a cell lysate from a tissue of a human to bind the DNA **fragment to wild-type p53** present in the cell lysate; and detecting the binding of the **p53-specific-binding DNA fragment to wild-type p53**.

Brief Summary Text - BSTX (15):

In yet another embodiment a double-stranded DNA **fragment is provided which comprises a p53-specific-DNA binding site, wherein the fragment** comprises more than one monomer repeat of the sequence 5'-RRRCWWGYYY-3' and wherein the fragment is covalently attached to an insoluble polymeric support.

Brief Summary Text - BSTX (17):

In yet another embodiment of the invention a method is provided for identifying compounds which specifically bind to p53-specific DNA binding sequences, comprising the steps of: contacting a **p53-specific DNA binding fragment** immobilized on a solid support with a test compound to bind the test compound to the DNA fragment; and determining the amount of test compound which is bound to the DNA fragment.

Brief Summary Text - BSTX (18):

In even another embodiment of the invention a method is provided for identifying compounds which specifically bind to p53-specific-DNA binding sequences, comprising the steps of: contacting a **p53-binding DNA fragment** immobilized on a solid support with both a test compound and wild-type p53 protein to bind the wild-type **p53 protein to the DNA fragment; determining the amount of wild-type p53 protein which is bound to the DNA fragment, inhibition of binding of wild-type p53** protein by the test compound suggesting binding of the test compound to the p53-specific DNA binding sequences.

Brief Summary Text - BSTX (23):

In another embodiment of the invention a method is provided of diagnosing tumor-inducing or hyperplasia-inducing strains of human papilloma virus (HPV) comprising: contacting cells or cell extracts of patients suspected of being infected by HPV with a **p53-specific binding DNA fragment; and detecting the amount of wild-type p53 in said cells or cell extract which binds to said DNA fragment, absence of bound p53** indicating infection by strains of HPV which sequester p53.

Drawing Description Text - DRTX (2):

FIG. 1A. Screening for **fragments bound by p53** using an immunoprecipitation assay. Panel 1 contains the hFosAva2 clone; panel 2, 772 C.sub.BE ; panel 3, Lambda 5R; panel 4, a pool of clones with inserts of randomly cloned human genomic sequences. 772 C.sub.BE and Lambda 5R contain Hinfl **fragments (259 and 190 bp, respectively) which bound p53** relatively strongly (arrowheads). "C"-

control lane, containing 2% of the labelled DNA used in the binding reactions. "B"- bound DNA recovered from the immunoprecipitate. FIG. 1B. Tests for dependence on p53 and specific antibody. Cell lysates were produced by infection with vaccinia virus that did (+) or did not (-) contain an insert of wild-type p53 cDNA. Immunoprecipitation was performed with anti-p53 monoclonal antibodies (+) or normal mouse IgG (-).

Drawing Description Text - DRTX (3):

FIGS. 2A and 2B. Relative abilities of wild-type and mutant **p53 to precipitate fragment** A. "C"- control lanes, containing 2% of the labelled DNA used in the binding reaction, "B"- bound DNA recovered from the immunoprecipitate. FIG. 2A. Increasing quantities of wild-type and mutant 273.sup.his p53, affinity-purified from a baculovirus expression system, were used to precipitate labelled C.sub.BE fragments. FIG. 2B. Lysates from a vaccinia virus system (Vac) producing the wild-type (wt), mutant (175.sup.his), or no **p53 protein (-), were used to immunoprecipitate labelled C.sub.BE fragments**. Equivalent quantities of p53 were present in the wild-type and mutant p53 lysates, as assessed by Western blot. In the "Bac" lane, affinity-purified p53 produced in baculovirus-infected insect cells was used in place of the vaccinia-infected lysates.

Drawing Description Text - DRTX (5):

FIGS. 4A and 4B. Binding of various subfragments of **fragments A and B to p53** from vaccinia-infected cell lysates. FIG. 4A. Subfragments of fragment A (subclone 10d) were assayed by immunoprecipitation for their ability to bind wild-type p53 from vaccinia-infected cell lysates. Binding of at least 2% of the DNA added to the reaction was judged as a positive (+) result; lesser but significant binding was recorded as "+/-". Double Lines (=) denote fragment A sequences. Single lines (-) denote polylinker sequences of the vector, not originally present in fragment A (FIG. 1). Fragment 5mut1 had a G to T transversion at bp 120; 5mut2 had G to T transversions at bp 120 to 122. FIG. 4B. The fragment A (panels 1-4) and fragment B (panel 5) subfragments illustrated in FIG. 4A are labelled to the left of the bands. The "v" band in panel 4 corresponds to the 2.9 kb vector into which subfragment 6 was cloned. Subfragment 8 (panel 5) contained bp 104-238 of fragment B (see FIG. 3B). Control lanes (C) contained 2% of the labelled fragments used in the binding assays (B).

Drawing Description Text - DRTX (8):

FIG. 6B shows Southern blot analysis of transfected clonal lines. The exogenous **p53 gene was present on a 1.8 kb BamHI fragment**. The endogenous p53 gene gave rise to a 7.8 kt BamHI fragment. Other sized fragments presumably arose by rearrangements.

Drawing Description Text - DRTX (11):

FIGS. 8A and 8B. Isolation of **human genomic sequences which bound to p53**.

Drawing Description Text - DRTX (12):

FIG. 8A. Experimental strategy used for isolation and analysis of human genomic DNA **fragments which bound to p53**.

Drawing Description Text - DRTX (13):

   FIG. 8B. Immunoprecipitation (IP) assays of cloned fragments. Clones of amplified and selected (AS) DNA were tested for the presence of **p53-binding fragments** by IP. For each clone, the bound DNA is shown in the B lane, adjacent to a control (c) lane containing 2% of the total end-labeled DNA used in the binding assay. In this representative experiment, eight binding fragments were identified, representing six unique genomic fragments. The inserts from the clones in lanes labeled, 2, 3, 5, 9, 10, and 11 contained **p53-binding fragments,** while the other lanes contained none. The clones in lanes 2 and 5 each contained two binding fragments.

Drawing Description Text - DRTX (18):

   FIG. 11B. Comparison of the ability of wild-type and mutant p53 to bind to the consensus dimer. In vitro translated p53 proteins were tested for the ability to bind the consensus dimer by IP. Two percent of the total DNA used for binding is shown in lane 1. Lane 7 shows binding to baculovirus-produced **human wild-type p53** protein. Lanes 2 to 6 show binding of in vitro translated wild-type and mutant p53 proteins. The mutant p53 proteins contained changes at codon 143 (val to ala), 175 (arg to his), 248 (arg to trp), and 273 (arg to his).

Drawing Description Text - DRTX (23):

   FIG. 13A. Relative DNA-binding abilities of various length concatemers of a pS3-binding sequence (PG.sub.n series), using an immunoprecipitation assay. Clones were cleaved by restriction endonucleases to extricate the concatemers, end-labelled, incubated with purified baculovirus-produced wild-type **human p53,** immunoprecipitated with anti-**p53 and protein A-Sepharose, and bound fragments** recovered and separated on a nondenaturing polyacryiamide gel. C, control lane, containing 2% of the labeled DNA used in the binding reactions. B, bound DNA recovered from the binding reactions.

Detailed Description Text - DETX (2):

   It is a finding of this invention that wild-type **p53 protein binds specific fragments** of human chromosomal DNA. Each of the fragments contains more than one monomer of the double-stranded motif 5'-RRRCWWGYYY-3' separated by 0 to 13 bp. Some of these sequences are found near origins of replication of certain animal viruses and animal cells. See Jelinek et al, Proc. Natl. Acad. Sci. USA, vol. 77, pp. 1398-1402 (1980). Four mutant forms of **p53 protein which are commonly found in human** tumors do not have the ability to bind to these sequences. Thus, a function of p53 may be mediated by its ability to bind to specific DNA sequences in the human genome.

Detailed Description Text - DETX (5):

   It has been found that **p53 will specifically bind to other sequences in the**

human genome with similar sequence motifs. Using a strategy coupling immunoprecipitation to "whole-genome PCR" (Kinzler, et al., Nucleic Acids Research, 17:3645-3653 (1989)), nineteen **human DNA fragments that bind to p53** have been identified. Each or the fragments contain a sequence which conforms to a dimer of the double-stranded motif 5'-RRRCWWGYYY-3', separated by 0 to 13 bp. These dimers directly mediate binding, as assessed by DNase I protection and methylation interference assays. The consensus dimers contain a striking symmetry, with four 5'-RRRCW-3' units oriented in alternating directions. A synthetic monomer containing the 10 bp consensus sequence is insufficient for binding, while the combination of two or more monomers bind strongly to wt p53, but negligibly to p53 mutants. Thus, more than one monomer appears to be required for binding. The spacing between monomers may be from 0 to 40 nucleotides, although all natural binding sites isolated have spacings of less than 15 nucleotides. The symmetry of the four half-sites within the consensus dimers suggests that p53 interacts with DNA as a tetrameric protein. The eighteen unique clones shown in FIG. 10 allow the identification of adjacent genes which may be regulated by p53 and may mediate its growth-suppressive action.

Detailed Description Text - DETX (8):
   Based on the sequence information of the **p53 specific-DNA-binding fragments,** a number of diagnostic and therapeutic methods have been devised. According to one such method, cell lysates are tested for the presence or absence of wild-type p53 by virtue of its specific DNA binding ability. As it is known for various cancers and stages of cancers that one or both of the p53 alleles in tumor tissues can be mutant, testing for the presence or absence of wild-type p53 protein can provide diagnostic and prognostic information regarding a tumor and the patient. The cells to be tested are typically isolated from a tissue suspected of being neoplastic. Preferably the tissues are carefully prepared and isolated so that non-neoplastic tissues are nor mixed with the neoplastic tissues, which can confound the analysis. Means for separating neoplastic tissues from non-neoplastic tissues are known in the art and include dissection of paraffin or cryostat sections, as well as use of flow cytometry. A cell lysate can be prepared from the tumor tissue according to any method known in the art. The cell lysate is then incubated with DNA **fragments which are known to bind the wild-type p53** protein, under conditions which are conducive to such DNA/protein interactions. Alternatively, a histological sample can be analyzed by incubation with DNA fragments, as described for cell lysates.

Detailed Description Text - DETX (11):
   According to another embodiment of the invention, after incubation of **p53 with specific binding DNA fragments** all components of the cell lysate which do not bind to the DNA fragments are removed. This can be accomplished, among other ways, by employing DNA fragments which are attached to an insoluble polymeric support such as agarose, cellulose and the like. After binding, all non-binding components can be washed away, leaving p53 bound to the DNA/solid support. The p53 can be quantitated by any means known in the act. It can be determined using an immunological assay, such as an ELISA, RIA or Western blotting.

Detailed Description Text - DETX (12):

The diagnostic assay of the present invention has applicability not only
with regard to cancers which are known to involve mutation Of **p53, but also
with regard t human** viruses such as human papilloma virus (HPV). HPV protein
E6 binds tightly to wild-type but not mutant p53. See Werness et al., Science,
248, 76-69 (1990). This tight binding is likely to block the interaction of
p53 with its specific DNA binding sequences. By testing cells or cell extracts
suspected of being infected with potentially tumor-inducing or
hyperplastia-inducing strains of HPV or possibly other viruses, infected cells
can be identified, because the E6 protein of the infected cells will have
sequestered the wild-type p53, rendering it unable to bind to its specific DNA
binding sequences. Such assays may be performed on cell extracts or on
histological specimens.

Detailed Description Text - DETX (13):

According to the present invention a method is also provided of supplying
wild-type p53 function to a cell which carries mutant p53 alleles. The
wild-type p53 gene or a part of the gene may be introduced into the cell in a
vector such that the gene remains extra-chromosomal. In such a situation the
gene will be expressed by the cell from the extrachromosomal location. If the
mutant p53 genes present in the cell are expressed, then the wild-type **p53 gene
or gene portion** should be expressed to a higher level than that of the mutant
gene. This is because the mutant forms of the protein are thought to
oligomerize with wild-type forms of the protein. (Eliyahu et al., Oncogene,
vol. 3, p. 313, 1988.) If a gene **portion is introduced and expressed in a cell
carrying a mutant p53 allele, the gene portion should encode a part of the p53**
protein which is required for non-neoplastic growth of the cell. More
preferred is the situation where the wild-type p53 gene or a part of it is
introduced into the mutant cell in such a way that it recombines with the
endogenous mutant p53 gene present in the cell. Such recombination would
require a double recombination event which would result in the correction of
the p53 gene mutation. Vectors for introduction of genes both for
recombination and for extrachromosomal maintenance are known in the art and any
suitable vector may be used.

Detailed Description Text - DETX (15):

According to the present invention compounds which have p53 activity are
those which specifically complex with a p53-specific DNA binding site.
Wild-type **p53 is one such compound, but portion of p53** which retain the ability
to bind to p53-specific binding sites be used. Oligonucleotides and
oligonucleotide containing nucleotide analogs are also contemplated among those
compounds which to complex with a p53-specific DNA binding site. Although
applicants do not wish to be bound by any particular theory, it is believed
that oligonucleotides bind double-stranded DNA to form triplexes. Such
triplexes have been shown to block transcription of certain gene, as well as
protect the DNA binding sites from the action of enzymes such as DNA
methylases. Although originally such oligonucleotitides were thought to
require only or predominantly pyrimidines (cytosine and thymine), purines have
also successfully been incorporated into triplex forming oligonucleotides.
Particular oligonucleotides which may be used include: nucleotides 140-162 of

SEQ ID NO:2, nucleotides 128-158 of SEQ ID NO: 1, nucleotides 114-123 of SEQ ID NO: 1, or portions thereof having at least ten nucleotides.

Detailed Description Text - DETX (19):
   Double-stranded DNA **fragments which comprise a p53**-specific DNA binding site and are attached to an insoluble polymeric support are also contemplated by this invention. The support may be agarose, cellulose, polycarbonate, polystyrene and the like. Such supported fragments may be used in screens to identify compounds which bind to p53-specific DNA binding sites. Similarly, such supported fragments may be used to perform diagnostic tests on cell lysates from suspected tumor tissues. They may also be used in assays used to screen potential chemotherapeutic agents, as discussed infra.

Detailed Description Text - DETX (20):
   Although any method can be employed which utilizes the p53-specific DNA binding sites of the present invention, two particular methods are disclosed for screening for additional compounds that bind to p53-specific DNA binding sites. According to one method a test compound is incubated with a supported DNA fragment, as described above. The amount of test compound which binds to the supported DNA fragment is determined. This determination can be performed according to any means which is convenient. For example, the amount of a compound which can be removed after incubation with the supported fragment can be compared to the amount originally applied. Alternatively, the test compound can be labelled and the amount which binds to the supported fragment can be assayed directly. In order to render this screening method more specific, soluble DNA **fragments which do not contain the p53** DNA binding sequence can be added to the incubation mixture. The soluble **fragments would not have me ability to specifically bind to p53** wild-type protein.

Detailed Description Text - DETX (21):
   According to another screening method for compounds to simulate the specific DNA binding activity of **p53, test compounds are incubated with supported DNA fragments** as described above. However, in this method wild-type p53 protein is also added to the incubation mixture. The amount of **p53 protein which binds to the DNA fragment** is measured using methods as described above. The amount of p53 protein bound is compared to the amount which binds in the absence of the test compound. Any diminution of p53 binding which results from the presence of the test compound is presumptively due to the competition of the test compound with **p53 for the specific DNA binding sites of the supported fragments**. Direct binding of the test compound to the binding site fragments can be confirmed using the assay described above.

Detailed Description Text - DETX (24):
   Compounds which have p53-specific DNA-binding activity, including wild-type **p53 protein, polypeptides corresponding to portions of wild-type p53** protein, oligonucleotides and oligonucleotide containing nucleotide analogues, as well as other organic molecules can also be administered to humans and animals as a pharmaceutical and therapeutic composition. Effective amounts will be

administered to cause neoplastic cells to become less aggressively neoplastic
or even to stop the growth of the neoplastic cells entirely. Generally, such
amounts will be in the range of 10 ng to 10 .mu.g per dose per person or other
animal. The therapeutic compounds can be prepared in any conventional
pharmaceutical excipient, such as physiological saline or other physiologically
compatible aqueous buffer. Typically, the compounds will be administered by
injection, either intravenous or intramuscular. However, other administration
methods as are known in the art and may be used to administer the compounds of
the present invention.

Detailed Description Text - DETX (30):
   In another embodiment of the invention, oligonucleotides can be isolated
which restore to mutant p53 proteins the ability to bind to The consensus
binding sequence or conforming sequences. Mutant p53 protein and random
oligonueleotides are added to a solid support on which **p53-specific-binding DNA
fragments** are immobilized. Oligonucleotides which bind to the solid support
are recovered and analyzed. Those whose binding to the solid support is
dependent on the presence of the mutant p53 protein are presumptively binding
the support by binding to and restoring the conformation of the mutant protein.

Detailed Description Text - DETX (40):
   Each clone was digested with an appropriate restriction endonuclease,
end-labelled with .sup.32 P, and incubated with p53 protein from a lysate of
cells infected with a recombinant vaccinia virus expressing p53 protein.
Labelled DNA **fragments which bound to p53** were then recovered by
immunoprecipitation with monoclonal antibodies against p53. Of the more than
1400 restriction **fragments tested, only two bound reproducibly to p53** under the
experimental conditions used: a 259 basepair Hinfl fragment (fragment A) of
clone 772 C.sub.BE (Panel 2, FIG. 1A), and a 190 basepair Hinfl fragment
(fragment B) of clone Lambda 5R (Panel 3, FIG. 1A); these fragments bound to a
far greater extent than any of the other labelled fragments of larger or
smaller size present in the same assay mixes.

Detailed Description Text - DETX (42):
   This example demonstrates that the immunoprecipitation **fragment A is
dependent on both p53** protein and anti-p53 antibodies.

Detailed Description Text - DETX (44):
   Lysates from cells infected with wild-type vaccinia virus (devoid of **p53)
were not able to specifically immunoprecipitate fragment** A (FIG. 1B).
Similarly, the detection of the precipitation of **fragment A was dependent on
the presence of anti-p53** antibodies (FIG. 1B). The binding was evident in
lysates prepared from either human HeLa cells or monkey BSC40 cells infected
with vaccinia virus and expressing wild-type p53 (FIG. 1B).

Detailed Description Text - DETX (45):
   Affinity-purified baculovirus-produced wild-type p53 protein was substituted

for the vaccinia-infected cell lysates in the immunoprecipitation assay and found to bind fragment A strongly (FIG. 2A). This suggested that the binding to **fragment A was an intrinsic property f the p53** polypeptide and not dependent on other factors present in the vaccinia virus-infected cell lysates.

Detailed Description Text - DETX (47):

The example demonstrates that **p53 mutant proteins found in human** tumors fail to bind to fragment A.

Detailed Description Text - DETX (48):

Increasing quantities of wild-type and mutant 273.sup.his p53 protein, affinity purified from a baculovirus expression system, were used to immunoprecipitate labelled fragments from C.sub.BE. See FIG. 2A. The proportion of **fragment A bound to wild-type p53** protein increased in tandem with the amount of p53 added to the assay mixture. (FIG. 2A) In contrast, fragment A did not specifically bind to a mutant form of p53 (273.sup.his) protein even at the highest p53 protein concentration used. The 273.sup.his mutation is the most common **p53 mutant identified in human** tumors. Another p53 mutant (175.sup.his) protein commonly found in human tumors also failed to bind to fragment A (FIG. 1B).

Detailed Description Text - DETX (50):

This example defines the particular sequences within **fragment A that allow it to bind to wild-type p53** protein.

Detailed Description Text - DETX (52):

One primer for each PCR was labelled with .sup.32 P at the 5' end with T4 polynucleotide **kinase** in a 5 1.mu.l reaction, and the **kinase** inactivated at 70.degree. C. for 5 min. PCR contained 350 ng of each of the appropriate primers and approximately 50 ng plasmid template in a 50 .mu.l reaction, using 25 cycles and the PCR conditions specified in Baker SJ, et al., Cancer. Res., 50:7717 (1990). The products were extracted with phenol and chloroform, ethanol-precipitated, and dissolved in 3 mM Tris, 0.2 mM EDTA prior to binding. Subfragment 1 contained bp 1 to 425 of subclone 10d of **fragment** A (FIG. 3A); subfragments 1a, 1b, 1c, 1d, and 1e were generated by digestion of subfragment 1 with BamHI, Mbol, HindIII, HindIII, and BamHI, respectively, from **fragment** 1. Subfragment 2, contained bp 283 to 425. Subfragment 3a was generated by digestion of subfragment 3 (bp 106 to 294) with Hae III. Subfragment 4a was produced from subfragment 4 (gp 1 to 141) by Hind III digestion. Subfragments 5a and 5b were products of the HaeIII digestion of subfragment 5 (bp 87 to 141). "Mutant" subfragments 5mut1 and 5 mut2 were produced using primers P3 ml (5'-GAAAGAAAAGGCAAGGCCAGGAAAGT-3') and P3mut2 (5'-GAAAGAAAAGGCAAGGCCATTAAAGT-3') and were identical to subfragment 5 except for the positions underlined in the primers. Subfragment 6 contained bp 106 to 138, and the insert was excised by restriction with HindIII and BamHI to generate 6a or with HindIII and EcoRI to generate 6b. Subfragment 3, including basepairs 106 to 294 (FIG. 4B, panel 2) bound well to **p53** as did subfragment 4, containing basepairs 1 to 141 (FIG. 4B, panel 3). This and similar assays done

with additional subfragments (FIGS. 4A and 4B) localized the critical sequences to Basepairs 106 to 141. This segment contained three repeats of the sequence TGCCT (FIG. 3A). Digestion of subfragment 3 with HaeIII (cleaving between bp 125-126 and removing two of the TGCCT repeats) greatly reduced this binding (FIG. 4B, subfragment 3A, panel 2), suggesting that a critical sequence lay at or near this restriction site and that a single TGCCT repeat was not sufficient for binding. Additional subfragments were tested (#5, bp 87 to 141, FIGS. 4A and 5B; #6, bp 106 to 138, FIGS. 4A and 4B, panel 4), and it was established that a 33 bp insert containing three TGCCT repeats provided binding capability.

Detailed Description Text - DETX (54):
   This example demonstrates that certain G residues are critical for binding of **p53 to fragment** A.

Detailed Description Text - DETX (58):
   This example defines the region of **fragment B which is important for p53** binding.

Detailed Description Text - DETX (61):
   This example shows that expression of the wild-type **p53 gene in human** colorectal carcinoma cells dramatically inhibits their growth and that a mutant **p53 gene cloned from a human** colorectal carcinoma was incapable of exerting such inhibition.

Detailed Description Text - DETX (69):
   The conclusions made from the above experiments are dependent on the assumption that p53 protein was produced in the transfected cell lines. Clones containing exogenous mutant p53 sequences produced p53 mRNA at a concentration 1.5 to 3.5 times higher than that produced by the endogenous p53 gene (FIGS. 6A and 7A). Immunoblot analysis showed that there was a concomitant small increase in p53 protein expression in the transfectants (1.5- to 3-fold) compared to the untransfected cells. However, this increase was difficult to measure quantitatively, since these cells produced significant amounts of endogenous p53 protein that (unlike endogenous p53 mRNA) could not be distinguished from that produced by the vectors. To confirm that transfected **human cells expressed p53** protein from our constructs, we studied an additional colorectal carcinoma cell line (RKO). RKO cells were obtained through the generosity of M. Bratrain. Although RKO cells did not contain a mutation within the susceptible p53 coding sequences, i.e., exons 5-9, they expressed low concentrations of p53 mRNA compared to normal colorectal mucosa or the other lines studied and did not produce detectable amounts of protein.

Detailed Description Text - DETX (70):
   Results of colony formation assays in transfected RKO cells were similar to those in SW480 and SW837 cells. Colony formation by wild-type p53 gene transfectants occurred with a tenfold decrease in efficiency compared to the mutant p53 construct (Table 1). Immunocytochemical detection of p53 protein in transfected RKO cells was done as follows: approximately 5.times.10.sup.4 cells

were cytocentrifuged onto polylysine-coated slides, fixed for 10 min in formalin, and permeabilized for 5 min in 0.5% Triton X-100. A mouse monoclonal antibody against **human p53** protein (Ab1801) in combination with the ABC immunoperoxidase system (Vector Laboratories), was used for immunocytochemical detection of p53 protein (Banks, et al., Eur. J. Biochem. 159, 529 (1986)). Ten to 20 randomly selected microscopic fields were analyzed per slide. These observations are consistent with the greater stability of mutant compared to wild-type p53 protein noted previously (C. A. Finlay et al., Mol. Cell Biol. 8, 531 (1988)). However, transient mRNA expression was also significantly lower in the SN3 transfectants compared to the SCX3 transfectants at 48 and 96 hours, supporting the idea that RKO cells expressing wild-type p53 were at a selective disadvantage compared to those producing mutant p53 products.

Detailed Description Text - DETX (76):
   This example demonstrates the identification of human genomic **fragments that can bond to wt p53** protein in vitro.

Detailed Description Text - DETX (78):
   Following the outline in FIG. 8A., we tested the inserts of 530 clones for binding to p53. Restriction fragments of the clones were end-labeled and incubated with purified **human wt p53** protein produced in baculovirus-infected cells.

Detailed Description Text - DETX (79):
   Whole-genome PCR was performed as previously described, except that only one oligonucleotide (5'-GAGTAGAATTCTAATATCTC-3') was used for amplification (Kinzler, et al. (1989), Nucleic Acids Research, 17:3645-3653, and Kinzler, et al. (1990), Molec. Cell. Biol., 10:634-642). Two hundred ng of "cateh"-linked human genomic DNA were incubated with 100 ng of baculovirus-produced **human wt p53** purified as described (Friedman, et al. (1990), Proc. Natl. Acad. Sci. U.S.A., 87:9275-9279), and immunoprecipitated as described below. After 4 rounds of IP and PCR, the AS DNA was cleaved with Eco RI and cloned into either the vector Lambda Zap II or pBluescript II SK+ (Stratagene). Individual clones were picked at random and tested for p53 binding. In panel B, cloned plasmid DNA samples were cleaved with Eco RI and end-labeled by Klenow fill-in. For IP (McKay, et al. (1981), J. Mol. Biol, 145:471-479), ten ng of DNA were incubated with 100 ng of baculovirus-produced **human wt p53** and 100 ng of poly dl-dC at 4.degree. C. for 30 minutes in 100 .mu.l of "DNA-binding buffer" containing 100 mM NaCl, 20 mM Tris, pH 7.0, 10% glycerol, 1% NP40, and 5 mM DTT. DNA **fragments bound to p53** were complexed to antibodies by the addition of 8 .mu.l containing 400 ng each of anti-p53 antibodies pAb421 and pAb1801, both obtained from Oncogene Science, and incubated for 30 minutes at 4.degree. C. The DNA-binding buffer containing 1.5 mg protein A precipitated following the addition of 26 .mu.l of DNA-binding buffer containing 1.5 mg protein A Sepharose and 10 .mu.g of poly dl-dC and mixing at 4.degree. C. for 30 minutes. After removal of the supernatant, the immunoprecipitate was washed twice with 1 ml of DNA-binding buffer. Bound DNA was purified by treatment with SDS and proteinase K at 48.degree. C. for 30 minutes. extracted with phenol and chloroform, precipitated with ethanol, separated by electrophoresis on a 10% nondenaturing polyacrylamide gel, and

autoradiographed.

Detailed Description Text - DETX (80):

Twenty-three of the clones were found to contain **fragments that b und to p53**. Examples of the IP experiments are shown in FIG. 8B. Clone S61 (lanes 11B,C) contains a single genomic **fragment of 202 bp which bound to p53**. Clone N2 contained five **fragments, only one of which (357bp) bound to p53** (lanes 10B,C). Other examples of **p53-binding fragments** were obtained, and each of these was subcloned for further analysis. In contrast, we found that none of over 1000 clones containing unselected human DNA inserts of similar size bound to p53 using the IP assay. Thus, the whole-genome PCR procedure significantly enriched for p53-binding sequences.

Detailed Description Text - DETX (82):

This example demonstrates the localization of **p53 contacts with bound DNA fragments**.

Detailed Description Text - DETX (83):

Localization of the regions bound by p53 was obtained by DP or MI assays using the subcloned DNA fragments as probes. For MI, the **fragments were methylated at G residues and bound to p53** (FIG. 9). Methylation of G residues critical for p53 binding resulted in interference with IP. For example, methylation at nucleotides, 217, 22, 227 to 229, and 233 of the 248 bp insert from clone 11B3 completely interfered with the binding of this **fragment to p53** (FIG. 9, footprint 2). When the opposite strand was analyzed, interference was observed at the G residues corresponding to nucleotides 219, 223, 224, 230, 235, and 236 (FIG. 9, footprint 1). For DP, labelled DNA fragments were first subject to IP, then incubated with various amounts of DNase I. For clone N22, p53 binding provided protection against DNase I cleavage at residues 187 to 211 (FIG. 9, footprint 9). MI showed interference by G residues only within the region protected by DNase I (FIG. 9, footprint 10). Other examples of DP and MI mapping are shown in FIG. 9. **p53-binding DNA fragments** were subcloned and labeled on one end, gel-purified and subjected to DP or MI mapping. For MI, 10 ng of DNA were incubated in 200 .mu.l of 50 mM Na-cacodylate, 1 mM EDTA, pH 8.0 and 5 .mu.l of 10% dimethylsulfate/90% ethanol for 5 minutes at 20.degree. C. to methylate G residues. Fifty .mu.l containing 1.5 m Na-acetate, 1 M .beta.-mercaptoethanol and 60 .mu.g of glycogen were added. The mixture was ethanol-precipitated, washed, and resuspended in 5 .mu.l of 3mM Tris, 0.2 mM EDTA, pH 7.5, and allowed to bind to wild-type p53 as described in the legend to FIG. 1. After IP and DNA purification, the samples were incubated with 100 .mu.l of 1 M piperidine at 90.degree. C. for 30 minutes. The samples were then dried under vacuum and separated electrophoretically on a 6% polyacrylamide sequencing gel. The control DNA samples were carried through all incubations except no p53 was added. For these control samples, the protein A Sepharose pellets were treated with SDS and proteinase K without removal of the supernatants (which contained the labeled DNA in the absence of p53).

Detailed Description Text - DETX (84):

For DP assays, end-labeled DNA fragments were immunoprecipitated as described in the legend to FIG. 8. The protein A Sepharose pellets were incubated for two minutes at 25.degree. C. with 200 ng DNase I in 5 mM MgCl.sub.2. After purification of the DNA, as described above, samples were separated by electrophoresis on sequencing gels and loaded as described above for MI. MI was performed on all 18 genomic DNA **fragments which bound to p53**. DP assays were performed on 13 fragments and the regions of protection uniformly coincided with those indicated by the MI assays.

Detailed Description Text - DETX (94):

This example demonstrates that intact **p53 can activate expression in human** cells.

Detailed Description Text - DETX (95):

We first made reporter plasmids (PG.sub.n -CAT series) containing part of the polyomavirus early promoter and the CAT gene located downstream of DNA sequences which could bind to **p53** in vitro (FIG. 8). For the CAT reporters, concatemers of the **p53**-binding region of C.sub.BE were formed by ligation of complementary oligonucleotides, ligated into the EcoRV site of pBluescript II SK+ (Stratagene) to form the PG.sub.n and MG.sub.n series. The BglII-BamHI **fragment** of pPyOlCAT (Murakami, et al. (1990) Oncogene, 5:5), containing the polyomavirus early promoter and the CAT gene coding region, was ligated into the BamHI sire of the PG.sub.n and MG.sub.n series clones to form the PG.sub.n -CAT and MG.sub.n -CAT series, and the orientation of the inserts characterized by restriction enzyme analysis. The PG.sub.9 -MG.sub.n -CAT and PG.sub.13 -MG.sub.n -CAT series were formed by excising the HindIII-SalI **fragments** of PG.sub.9 -CAT and PG.sub.13 -CAT, blunt-ending, attaching XbaI linkers, and ligating into the XbaI site of the MG.sub.n -CAT series plasmids (where n=I, 5, 10, and 15). For the yeast .beta.-galactosidase reporter plasmids, PG and MG sequences were ligated as SalI-SmaI **fragments** to the SalI and filled-in XhoI sites of pCZ (Buchanan, et al. (1988), Mol. Cell Biol., 8:50806). The construction of the **p53**-wt expression construct has been described (Baker, et al. (1990), Science, 249:912); the mutant expression plasmids were constructed similarly from the previously described cDNA plasmids (Nigro, et al. (1989), Nature, 342:705, and Kern, et al. (1991), Oncogene, 6:131), or in the case of the engineered **phosphorylation** site mutants, by in vitro mutagenesis (Altered Sites, Promega) with verification by sequencing. The construction of the yeast **p53** expression vectors based on pRS314 has been described (Nigro, et al., Mol. Cell Biol. (in press)).

Detailed Description Text - DETX (96):

For the p53 binding sequences, we used a series of concatemers of the oligonucleotide PG (5'-CCTGCCTGGACTTGCCTGG-3'). This contained the binding region of plasmid C.sub.BE, previously shown to bind p53 in vitro. The reporter and an expression vector coding for the intact **human wild-type protein (p53**-wt) (FIG. 12B), were transfected together into the human colorectal cancer cell line HCT 116. This line makes low amounts of apparently wild-type p53 protein.

Claims Text - CLTX (17):
  adding (a) a p53 protein which is encoded by a mutant gene found in a cancer patient and (b) a preparation of random oligonucleotides to (c) a **p53-specific-binding DNA fragment immobilized on a solid support, to bind p53 protein to said p53-specific-binding DNA fragment immobilized on a solid support, wherein said p53-specific-binding DNA fragment** contains more than one monomer of the double stranded motif RRRCWWGYYY; and

Claims Text - CLTX (18):
  recovering oligonucleotides from said preparation which bound to **p53 which bound to said p53-specific-binding DNA fragment** immobilized on the solid support.

Claims Text - CLTX (36):
  adding (a) a p53 protein which is encoded by a mutant gene found in a cancer patient and (b) a preparation of random oligonucleotides to (c) a **p53-specific-binding DNA fragment immobilized on a solid support, to bind p53 protein to said p53-specific-binding DNA fragment immobilized on a solid support, wherein said p53-specific-binding DNA fragment** contains more than two monomers of the monomer sequence TGCCT; and

Claims Text - CLTX (37):
  recovering oligonucleotides from said preparation which bound to **p53 which bound to said p53-specific-binding DNA fragment** immobilized on the solid support.

Other Reference Publication - OREF (6):
  Mercer, et al., "Negative Growth Regulation in a Glioblastoma Tumor Cell Line that Conditionally Expresses **Human Wild-Type p53**", Proc. Natl. Acad. Sci. USA, 87:6166-6170(1990).

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	87	dna adj (pk or activated adj (protein adj kinase$1 or pk))	USPAT; US-PGPUB	2003/06/04 14:11
2	L2	25	1 same (substrate$ or peptide$)	USPAT; US-PGPUB	2003/06/04 14:18
3	L3	19	1 same (assay$8 or detect$8 or quantit$8)	USPAT; US-PGPUB	2003/06/04 14:20
4	L4	6232	p53	USPAT; US-PGPUB	2003/06/04 14:23
5	L5	505	4 near6 (fragment$4 or peptide$ or portion$)	USPAT; US-PGPUB	2003/06/04 14:27
6	L6	971	4 near4 human	USPAT; US-PGPUB	2003/06/04 14:27
7	L7	279	5 and 6	USPAT; US-PGPUB	2003/06/04 15:11
8	L8	471	4 same (phosphorylat$ or kinase$1 or termin$8) same (fragment$4 or peptide$ or portion$)	USPAT; US-PGPUB	2003/06/04 15:14
9	L9	130	8 and 7	USPAT; US-PGPUB	2003/06/04 15:14
10	L10	12	5 and 1	USPAT; US-PGPUB	2003/06/04 16:21

PGPUB-DOCUMENT-NUMBER: 20030022263

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030022263 A1

TITLE: ATM kinase modulation for screening and therapies

PUBLICATION-DATE: January 30, 2003

US-CL-CURRENT: 435/15

APPL-NO: 10/ 024123

DATE FILED: December 17, 2001

RELATED-US-APPL-DATA:

  child 10024123 A1 20011217

  parent division-of 09400653 19990921 US PATENTED

  child 09400653 19990921 US

  parent continuation-in-part-of 09248061 19990210 US PENDING

[0001] This is a division of application Ser. No. 09/400,653, filed Sep. 21, 1999, which is a continuation-in-part of and claims the priority of U.S. application Ser. No. 09/248,061 filed Feb. 10, 1999. Each of these prior applications is hereby incorporated herein by reference, in its entirety.

[0001] This application claims the benefit of U.S. Provisional Application No. 60/218,498, filed Jul. 14, 2000, the content of which is hereby incorporated by reference.

PGPUB-DOCUMENT-NUMBER: 20020165218

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020165218 A1

TITLE: Materials and methods to potentiate cancer treatment

PUBLICATION-DATE: November 7, 2002

US-CL-CURRENT: 514/210.2, 514/217.05 , 514/217.06 , 514/242 , 514/252.01
, 514/252.03 , 514/252.05 , 514/256 , 514/84 , 514/85
, 544/182 , 544/232 , 544/238 , 544/243 , 544/319 , 544/328

APPL-NO: 09/ 941897

DATE FILED: August 28, 2001

RELATED-US-APPL-DATA:

 non-provisional-of-provisional 60229899 20000901 US

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of provisional U.S. Patent
Application No. 60/229,899, filed Sep. 1, 2000.

PGPUB-DOCUMENT-NUMBER: 20020090643

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020090643 A1

TITLE: COMPOSITIONS AND METHODS FOR MONITORING THE
             PHOSPHORYLATION OF NATURAL BINDING PARTNERS

PUBLICATION-DATE: July 11, 2002

US-CL-CURRENT: 435/7.1

APPL-NO: 09/ 258981

DATE FILED: February 26, 1999

CONTINUED PROSECUTION APPLICATION: This is a publication of a continued
prosecution application (CPA) filed under 37 CFR 1.53(d).

PGPUB-DOCUMENT-NUMBER: 20020019002

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020019002 A1

TITLE: Methods of monitoring enzyme activity

PUBLICATION-DATE: February 14, 2002

US-CL-CURRENT: 435/6

APPL-NO: 09/ 877919

DATE FILED: June 7, 2001

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60211313 20000613 US

US-PAT-NO: 6441158

DOCUMENT-IDENTIFIER: US 6441158 B1

TITLE: Oligomers that bind to ku protein

DATE-ISSUED: August 27, 2002

US-CL-CURRENT: 536/24.5, 536/23.1

APPL-NO: 09/ 223139

DATE FILED: December 30, 1998

PARENT-CASE:

  CROSS-REFERENCE TO RELATED APPLICATIONS

  This application claims benefit of U.S. provisional application No.
60/070,278, filed Dec. 31, 1997.

US-PAT-NO: 6387640

DOCUMENT-IDENTIFIER: US 6387640 B1

TITLE: ATM kinase modulation for screening and therapies

DATE-ISSUED: May 14, 2002

US-CL-CURRENT: 435/15, 435/194 , 435/252.3 , 435/320.1 , 435/325

APPL-NO: 09/ 248061

DATE FILED: February 10, 1999

US-PAT-NO: 6348311

DOCUMENT-IDENTIFIER: US 6348311 B1
See image for Certificate of Correction

TITLE: ATM kinase modulation for screening and therapies

DATE-ISSUED: February 19, 2002

US-CL-CURRENT: 435/5, 435/15

APPL-NO: 09/ 400653

DATE FILED: September 21, 1999

PARENT-CASE:

This application is a continuation in part of U.S. application No. 09/248, 061 filed 02/10/1999 pending.

US-PAT-NO: 6171857

DOCUMENT-IDENTIFIER: US 6171857 B1
See image for Certificate of Correction

TITLE: Leucine zipper protein, KARP-1 and methods of regulating
               DNA dependent protein kinase activity

DATE-ISSUED: January 9, 2001

US-CL-CURRENT: 435/325, 435/252.1 , 435/320.1 , 536/23.1 , 536/23.5
        , 536/24.3 , 536/24.31 , 536/24.33

APPL-NO: 09/ 173914

DATE FILED: October 16, 1998

PARENT-CASE:

   RELATED APPLICATIONS

   This application claims priority under 35 USC .sctn. 119(e) from U.S.
Provisional Patent Application Ser. No. 60/064,557 filed on Oct. 17, 1997,
entitled A NOVEL LEUCINE ZIPPER PROTEIN, KARP-1 AND METHODS OF REGULATING
DNA
DEPENDENT PROTEIN KINASE ACTIVITY. The content of the provisional application
is hereby expressly incorporated by reference.

US-PAT-NO: 6140058

DOCUMENT-IDENTIFIER: US 6140058 A

TITLE: Activation of p53 protein

DATE-ISSUED: October 31, 2000

US-CL-CURRENT: 435/7.1, 424/155.1 , 424/174.1 , 435/7.23 , 530/350 , 530/358

APPL-NO: 08/ 446668

DATE FILED: July 24, 1995

          FOREIGN-APPL-PRIORITY-DATA:
   COUNTRY   APPL-NO   APPL-DATE
   GB   9224784   November 26, 1992

PCT-DATA:
 APPL-NO: PCT/GB93/02438
 DATE-FILED: November 26, 1993
 PUB-NO: WO94/12202
 PUB-DATE: Jun 9, 1994
 371-DATE: Jul 24, 1995
 102(E)-DATE:Jul 24, 1995

US-PAT-NO: 6057104

DOCUMENT-IDENTIFIER: US 6057104 A

TITLE: Disruption of the mammalian Rad51 protein and disruption
                  of proteins that associate with mammalian Rad51 for
                  hindering cell proliferation

DATE-ISSUED: May 2, 2000

US-CL-CURRENT: 435/6, 435/196 , 530/350 , 536/23.2 , 536/23.5

APPL-NO: 08/ 964614

DATE FILED: November 5, 1997

PARENT-CASE:

  The present application is a continuation-in-part of and claims priority to
U.S. applications Ser. Nos. 08/758,280, filed Nov. 5, 1996. The disclosure
of the above application is herein incorporated by reference.